Kinetics of B cell development in adult mice

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1 Introduction

1.1 Immune system

The immune system endows the individual with a self-defence system that is essential for protection against the harms of pathogens. Functionally, to this end the immune system carries out two related tasks – recognition and response and can be qualitatively divided into two components – non-specific and specific. The non-specific component is known as innate immunity whereas the component with high degree of specificity as well as the remarkable property of memory is known as adaptive immunity. Innate immunity plays an important role in the first line of immune defence and involves the recognition of a broad range of so called PAMPs (pathogen associated molecular pattern) that are common to many pathogens. On the other hand, the adaptive immunity develops newly in every individual during maturation and involves so called B and T cells as major components. These two cell types belong to lymphocyte subset and are capable of recognizing different pathogens with highly specific receptors, display memory function and carry out humoral (antibody mediated) and cell mediated immune responses, respectively. Since B cells are the subject of the present work, in the following, these cells and their functions will be described in more detail.

1.2 B lymphocytes

B cells develop in the fetal liver or the bone marrow. During this phase they acquire unique antigen-binding receptors, that can bind to its nominal antigen very specifically. These receptors are known as B cell receptor (BCR) and are essentially membrane bound antibodies. Upon binding of the specific antigen to a BCR in conjunction with other stimuli, B cells differentiate into effector B cells. These effector B cells can be divided into two subsets: plasma cells that are antibody secreting cells, and memory B cells, that can be activated upon secondary encounter with the specific antigen, for example in case of secondary infection. Antibodies produced by plasma cells in secretory form constitute the major effector component of humoral immunity.

1.2.1 Structure of antibodies

B cells mainly function via their BCR that recognize a specific antigen. The soluble and secretory form of this protein is called immunoglobulin (Ig) or antibody (Ab) when the specificity is known. It is a tetrameric glycoprotein that consists of two heavy (H chain) and two light chains (L chain). These chains are linked via disulfide bonds and both heavy chains and both light chains are identical in one tetramer. The scheme of an IgG Ab is shown in Figure 1.1.

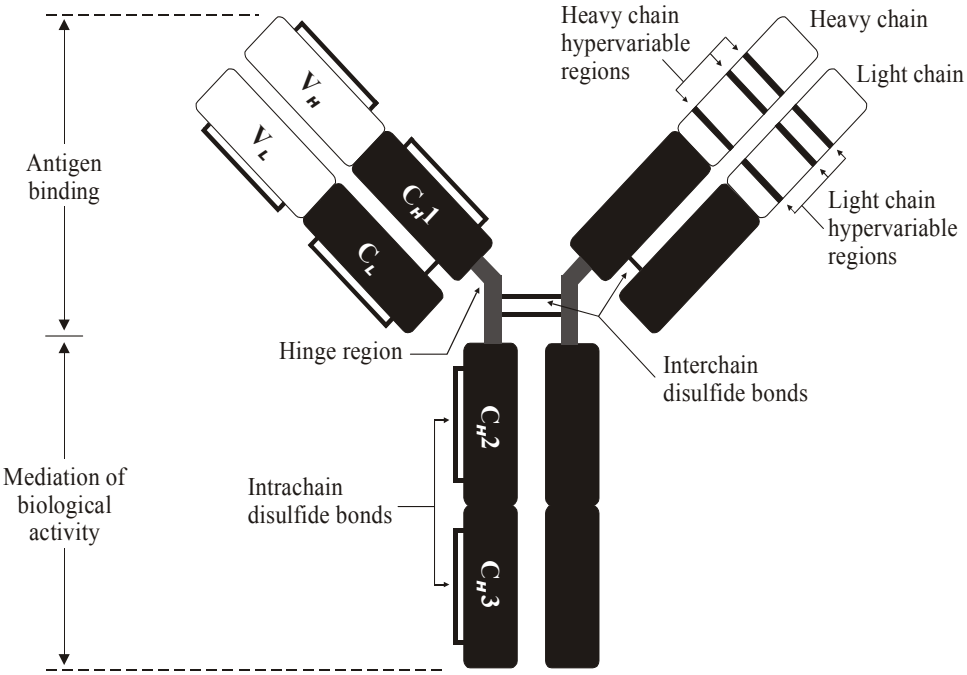

Figure 1.1 Schematic representation of an IgG molecule domain structure

An antibody is composed of two heavy and two light chains. Depending upon the heavy chain constant region, the antibody molecule can belong to one of the five isotypes – IgA, IgD, IgE, IgG and IgM. Each antibody contains either λ or κ light chain isotype. The hinge region provides steric flexibility and is found in IgD, IgG and IgA isotypes, whereas IgM and IgE isotypes contain an additional CH domain. Figure adopted from (Düber, 2004).

The chains in an antibody molecule are composed of a variable (V) and a constant (C) region. While the variable region of the two chains forms the antigen binding site that varies between each antibody molecule on different cells, the constant region is conserved in each of the five existing

immunoglobulin subsets (IgA, IgD, IgE, IgG and IgM). This constant part of the immunoglobulin is responsible for the effector function of the molecule. Enormous variability in the V region of antibody molecules are responsible for the high diversity in the antibody repertoire. On the basis of the amino acid sequence, each V region contains three highly variable stretches on each chain (see Figure 1.1). These stretches are called complementarity-determining region (CDR1-3) or hypervariable regions. Among these three CDRs the CDR3 is the most variable. The four regions flanking the CDRs are called framework regions (FR1-4), are less diverse and responsible to maintain the structure of the variable region. By pairing of the V regions of a heavy and a light chain (V_H and V_L) the CDRs are brought together to form the antigen (Ag)-binding site thus generating a complex that is complementary to its Ag. Generation of a diverse repertoire of such molecules takes place during the B cell development and maturation.

1.2.2 How to distinguish B cell subsets?

B cells, as other cells from the immune system can be differentiated into various subsets upon their different pattern of surface markers. Very common markers for the differentiation of those cells are the cluster of differentiation (CD) molecules. Each cell subset as well as each ontogenic state are characterized by a specific combination of these markers. In addition, the expression of intracellular molecules changes during differentiation and maturation as well. In Figure 1.2 the different markers during B cell development are shown. Besides their expression of surface markers, mature B cells can be furthermore divided into different subsets by function and anatomical location (see chapter 1.2.5).

1.2.3 B cell development

During embryonic stage, pluripotent hematopoietic stem cells (PHSCs) found in the fetal liver can give rise to B cells. Later on during the neonatal and adult stages, precursor B cells are generated from common lymphoid progenitors (CLPs) that originate from PHSCs (Hardy and Hayakawa, 2001) (Akingbemi et al., 2001)in the bone marrow. The development of B cells is controlled by interactions between these cells and the surrounding cytokine milieu produced by stromal cells (Vosshenrich et al., 2003; Vosshenrich et al., 2004) .It is tightly

regulated at different check-points. If the cell fails to overcome a check-point, it undergoes apoptosis.

		proB	preB-I	large preB-II type I	large preB-II type II	small preB-II	immature B	mature B
Ig-gene-rearrangement	**H:**	N.R.	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
	L:	N.R.	N.R.	N.R.	N.R.	$V_\kappa J_\kappa$	$V_\kappa J_\kappa$	$V_\kappa J_\kappa$
						$V_\lambda J_\lambda$	$V_\lambda J_\lambda$	$V_\lambda J_\lambda$
suerfacemarker	**B220**	+	+	+	+	+	+	+
	CD19	-	+	+	+	+	+	+
	c-kit	+	+	-	-	-	-	-
	AA4.1	+	+	+	+	+	+	-
	IL-7-R	+	+	+	+/-	-	-	-
	CD43	+	+	+	+/-	-	-	-
	CD25	-	-	+	+	+	+/-	-
	CD40	-	-	-	-	+/-	+	+
	CD23	-	-	-	-	-	-	+
Recombination	**RAG-1**	-	+	-	+/-	+	+ (protein)	-
	RAG-2	-	+	-	-	+	+ (protein)	-
	TdT	-	+	-	-	-	-	-
B cell receptor	**Igα, Igβ**	+	+	+/-	-	-	-	-
	Iμ	+	+	+	+	+	+	+
	SL	+	+	+	-	-	-	-
	cyto-μ	-	-	+	+	+	+	+
	κ^0	-	-	-	+	+	+	+
	κ/λ	-	-	-	-	-	+	+
	sIgM	-	-	-	-	-	+	+
	sIgD	-	-	-	-	-	+/-	+

Figure 1.2 Expression of B cell specific markers during its development.

H: heavy chain; L: light chain; N.R.: not rearranged; +: expression; -: no expression; protein: detectable only at protein level; Iμ: germline transcripts of the heavy chain locus; SL: surrogate light chain; Cyto-μ: cytoplasmic μ chain; κ^0: germline transcripts of κ light chain. Adapted from (Engel, 1999)

The earliest committed B cell precursors are known as proB cells that can be identified by the expression of B220 and the lack of CD19 (Ogawa et al., 2000)

that is expressed from later stage - preB-I stage - onwards as the main B cell marker (see Figure 1.2). In addition, AA4.1 is also a very common marker to distinguish progenitor B cells from other subsets in the bone marrow as it is expressed throughout the whole process of maturation and is lost on mature B cells (Hardy and Hayakawa, 2001).

The pre B cell stage can be separated into two main populations, containing dividing cells (large preB-II) and small resting preB cells (small preB-II; (Hardy et al., 1991; Tung et al., 2006)). In preB-I cells, the heavy chain of the BCR is rearranged. If this pairs effectively with the surrogate light chain it can be transported to the cell surface and is presented as pre-BCR. Non-matching heavy chains lead to either further rearrangement or to apoptosis of this cell. During small preB cells stage, the light chain of the BCR is arranged and by fitting this to the heavy chain the cell can further differentiate into the immature state in which they become independent of stromal factors in the BM (Wasserman et al., 1998).B cells migrate out of the BM at the immature stage and further mature in the spleen. All stages can be distinguished from each other by surface molecules as shown in Figure 1.2.

1.2.4 Heavy chain rearrangement

During the maturation of B cells, the B cell receptor (BCR) is generated in a well regulated manner. First, gene segments of the heavy chain are rearranged. to form the variable region (V) of the heavy chain. It is composed of different gene segments that need to be combined by somatic DNA recombination. The V region of the heavy chain contains a variable domain (V_H), a joining domain (J_H) and a diversity domain (D_H) that is located between V and J.

The three gene segments of the heavy chain variable region span a length of 1.5 mega bases (Mb) and heavy chain (IgH) itself is further extended to 3 Mb by the genes encoding the constant part of the BCR (Chowdhury and Sen, 2004).

The light chain does not contain any D, thus containing only V_L and J_L. Two different light chains exist in mice – κ and λ. They are functionally identical and only differ in their order of expression during ontogeny of a B cell (Roy, 2007). It

is that only one of these light chains can be used and presented on the surface at a complete BCR.

On DNA level, the V_H are located most distant from the constant domain compared to the other segments. In mice, the V_H locus can be divided into 15 gene families containing more than 100 V_H gene segments (Chevillard et al., 2002; Chowdhury and Sen, 2004). Recombination of different genes to form a heavy chain is divided into two steps. In the first step, one of 13 D_H gene segments and one of four J_H gene segments are recombined during preB-I stage. In the second step, one of the V_H genes is rearranged to the DJ_H for completion of the V_H region exon (see Figure 1.3).

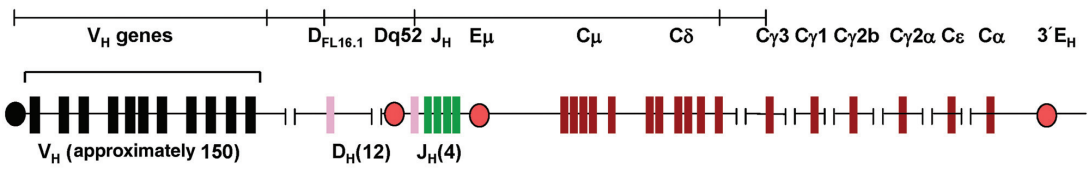

Figure 1.3 Organization of IgH locus

Location and order of IgH gene segments has been depicted. Black rectangles represent several VH genes, the 5'-most (DFL 16.1) and the 3'-most DH gene segments have been depicted by pink rectangles. Green and maroon rectangles represent the JH and constant chains respectively. Numbers in the parenthesis show the number of members belonging to the respective gene family. Three known *cis*-regulatory elements, the intronic enhancer Eμ, the Dq52 promoter, and the 3' locus control region are depicted as red ovals (Chowdhury and Sen, 2004).

This recombination process is tightly regulated and requires the expression of the recombination activating proteins RAG-1 and RAG-2 (Mombaerts et al., 1992; Shinkai et al., 1992). Due to a process called allelic exclusion most B cells express only a single heavy chain on their surface, although all cells contain two heavy chain alleles (Loffert et al., 1996; Melchers et al., 1999). One of the checkpoints of B cell development is the assembly of the generated heavy chain to a surrogate light chain (SLC) that consists of the proteins λ5 and VpreB. This whole complex is called pre-BCR. As soon as a proper heavy chain is able to bind to the SLC and can be presented at the cell surface together with other co-

receptors - Igα and Igβ (Reth et al., 2000), the cell proliferates and continues differentiation (Geier and Schlissel, 2006).

In the absence of a proper pre-BCR the RAG-expression is not switched off and the recombination of genes proceeds (Grawunder et al., 1995). Further development of the B cell is transiently stopped. This mechanism is one of the control mechanisms that screens for an appropriate heavy chain being able to bind to a light chain later on in development.

The rearrangement process results in a broad repertoire of different heavy chains and in high diversity that is called combinatorial diversity as it is due to the random selection of members of the gene segment family for recombination. Another reason for the highly diverse repertoire is the consequence of junctional diversity. This is due to the addition or deletion of nucleotides at the junctions between the different gene segments during the recombination process. While the so-called P-nucleotides are added during fetal and adult phase, so called N-nucleotides are only added in adult BM precursors and are not or very rarely found in case of B cells that develop during the fetal stage (Li et al., 1993).

It is known that in fetal liver, first the proximal V_H genes and later during rearrangement the distal V_H genes are used (Wasserman et al., 1998). It could be shown that pre-BCR complexes isolated from BM cells leads to proliferation, whereas the same pre-BCR induces the exit of the cycle in fetal liver. There is also some evidence, that the proximal V_H genes are used first by progenitors in the adult BM (Malynn et al., 1990; ten Boekel et al., 1997) but for one study this was only shown by isolating pools of BM cells from young mice (Malynn et al., 1990) so that there is still a risk of contamination of recirculating B cells. The other study only analysed a very low number of sequences (ten Boekel et al., 1997).

The analysis of acetylation of different V_H genes during rearrangement revealed that there are differently regulated subdomains. The most distal V_H domains are responding on IL-7, the genes lying in the middle can be activated by *v-abl* and the most proximal genes were found in an acetylated state as soon as the DJ_H recombination occurred (Chowdhury and Sen, 2001). It was proposed, that this

is also a hint for the usage of proximal V genes during early developmental stages of B cells in adult BM.

1.2.5 B cell maturation

Immature B cells express elevated levels of surface IgM, therefore this is a marker for immature B cells (Xing et al., 2009). After differentiation, these immature B cells migrate out of the BM and via the blood stream into peripheral sites like spleen or peritoneal cavity. In the periphery, the immature or transitional (T) B cells can be negatively selected against self-antigens. This represents another checkpoint for B cell maturation and can lead to apoptosis.

It was shown that in the periphery three different types of transitional B cell subsets (T1-3) could be defined by their expression of surface IgM, CD23 and AA4.1 (Allman et al., 2001). This led to the suggestion that also in periphery a stepwise B cell maturation might be taking place (Allman et al., 2001; Allman and Pillai, 2008; Loder et al., 1999). Interestingly, Teague and colleagues could show that the T3 B cells do not give rise to mature B cells, but rather differentiate away from the mature B cell subsets (Teague et al., 2007).

The mature B cells in the periphery can again be assigned to three different main subsets: B-1, B-2 or follicular (FO) and marginal zone (MZ) B cells. The classification can be determined according to their characteristics as size, cell surface phenotype, functional activity and anatomical location (Allman and Pillai, 2008; Martin and Kearney, 2001; Pillai et al., 2004).

B-2 cells are the key players in humoral immune defence during adoptive immune reaction. Their antibody repertoire is highly diverse as the BCR besides combinatorial and junctional diversity is undergoing somatic hypermutation (SHM) upon recognition of an antigen to develop higher affinity (affinity maturation). This mutational process is driven by a special enzyme - AID (activation induced cytidin desaminase) - which leads to single nucleotide replacements (see chapter 1.2.7). Upon appropriate selection, SHM leads to a further increase of antibody repertoire as the rearrangement process in the maturing precursor B cells already leads to. B cells originating from the B-2 subset show a defined pattern of surface markers ($CD19^{+}B220^{hi}IgM^{lo}IgD^{hi}CD43^{-}$

$CD21^{int}CD23^{hi}$) and require T cell help for their differentiation into effector B cell. This indicates that they can be activated to become antibody-secreting cells or memory B cells just in a T-dependent manner.

In contrast, MZ and B-1 B cells can be activated without T cell help. However, their antibody repertoire is more restricted as the repertoire of B-2 cells. MZ B cells can be mainly found in the marginal zone of the spleen and can be characterized by their expression patter as $CD19^{+}B220^{hi}IgM^{hi}IgD^{lo}CD43^{-}$ $CD21^{hi}CD23^{lo/-}$ cells. These cells can be activated by blood borne pathogens (Martin et al., 2001) and can thereupon differentiate into short-lived plasma cells (Amezcua Vesely et al., 2012). Furthermore, they were thought to be non-migrating, they rather act as shuttle for transportation of antigens as immune complexes from the marginal zone to B-2 cells in the B cell follicles of the spleen (Cinamon et al., 2008; Tse et al., 2012).

The other B cell subset, B-1 cells, can be characterized as $CD19^{hi}B220^{lo}IgM^{hi}IgD^{lo}CD43^{+}CD23^{lo/-}$ expressing cells. In the peritoneal cavity, one of their primary locations, most B-1 cells also express CD11b or Mac-1. Furthermore, this population can be subdivided due to their expression of CD5 into B-1a ($CD5^{+}$) and B-1b ($CD5^{-}$) cells (Martin and Kearney, 2001). While B-1a cells are crucial for the first line of defence and are defined as innate-like B cells, B-1b cells are thought to be involved in the adaptive immune response and can provide long-lasting T cell-independent IgM memory (Alugupalli et al., 2004). Both B-1 cell populations can be activated in a T cell independent manner and B-1a cells are the major source of natural IgM (for more details see chapter 1.3). In contrast to B-2 cells, B-1 cells are not thought to derive from CLP in the bone marrow in adult live but are generated from distinct precursors that can be found in fetal or neonatal live and in adult bone marrow. These precursors are defined as $Lin^{-}B220^{lo/-}CD19^{+}$ cells (Düber et al., 2009; Montecino-Rodriguez et al., 2006). Although both populations are self-renewing, the activity for B-1a progenitors decreases after birth, while the activity for B-1b progenitors is active in neonatal and adult bone marrow (Düber et al., 2009; Martin and Kearney, 2001).

1.2.6 Requirement of T cells

For their activation to differentiate into an antibody secreting plasma cell or a memory B cell, B-2 cells require direct contact with T cells, which takes place in the secondary lymphoid organs like spleen or lymph nodes. As naïve B cell, these cells express IgM and IgD. Depending on the cytokine-milieu that is available during the activation of a B cell, it is determined which kind of immunoglobulin it will express after differentiation. This process is called class switch as cells switch from expression of IgM/IgD to IgE, IgG or IgA (see 1.2.7, (Manis et al., 2002; Stavnezer et al., 2008)).

On the other hand, it was shown that B cells are required for the development of T cell zones in spleen and the accumulation of DCs and T cells in this organ (Ngo et al., 2001). Furthermore, B cells can trigger the activation of T cells by presenting antigens via MHCII on their cell surface. In addition, B cells express CD80 and CD86 and thus can bind to the agonistic CD28 on T cells and hence promote T cell activation. However, it is not necessary that B cells activate T cells directly. Natural IgM or other Igs themselves can guide the polarization of T cell response by promoting the recognition of pathogens by professional antigen presenting cells (APCs), for example dendritic cells (Rapaka et al., 2010).

As described shortly in this chapter, T cells and B cells have some influence on each other. Nevertheless, it is not clear yet, whether T cells are necessarily required for B cell development or only for their activation.

1.2.7 Class switch

During class switch, or isotype switch, the C_H region of an antibody is changed. This is important for the effector function of the antibody as different subclasses bind with different affinities to special receptors on the surface of other immune cells or on epithelial cells. According to the strength of the binding, the activation of these cells can be triggered.

Naïve B cells express IgM and IgD and in response to external signals, as cytokines released by T cells during activation of B cells class switch recombination (CSR) can be induced (Manis et al., 2002; Stavnezer et al., 2008). CSR depends on DNA rearrangement and recombination between Sμ

(switch region, that lies directly upstream of genes encoding μ heavy-chain domains) and S_x region, located upstream of genes encoding the heavy-chain domains of isotype x (IgA, IgE, IgG1, IgG2a/b, IgG3) (see Figure 1.4.). For CSR, double stranded DNA breaks (DSBs) are required. This is promoted by the activation-induced cytidine deaminase (AID) as it deaminats dC to dU in the S regions and the subsequent repair of these dU residues leads to DNA breaks ((Stavnezer et al., 2008) Figure 1.4).

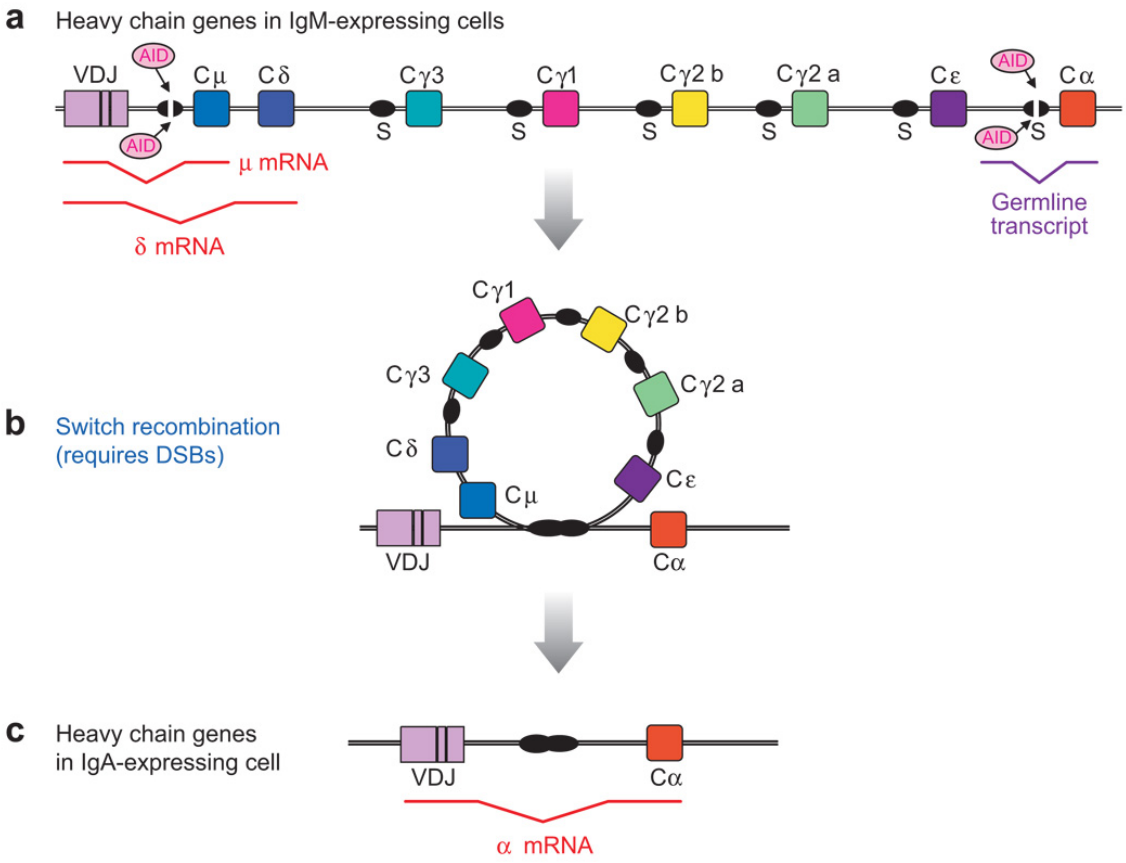

Figure 1.4 Diagram of Ig class switch recombination (CSR) from IgM to IgA

(*a*) The mouse IgH locus in B cells expressing IgM and IgD (by alternative RNA transcription/processing). During CSR, activation-induced cytidine deaminase (AID) deaminates dC residues in the top and bottom strands of transcriptionally active S regions (Sμ and Sα in the diagram shown), initiating a process that results in double-strand DNA breaks (DSBs) in both S regions and CSR by intrachromosomal deletion (*b*). (*c)* The IgH locus after CSR to IgA. Splicing diagrams of the μ, δ mRNAs and the germline α transcript are indicated below the diagram of the locus. Similar germline transcripts are induced from unrearranged Cγ,Cε, and Cα genes, depending on the cytokine stimulation received by the B cell (Stavnezer et al., 2008).

Nonhomologous end-joning of the DNA ends leads to excision of all DNA between the two switch regions and formation of a new immunoglobulin with the same variable region but a different C_H. Thus, it results in a newly formed immunoglobulin.

1.3 IgM

Immunoglobulins are important for the humoral immune response in human and mice. B cells that secrete these molecules can be distinguished by their different expression of surface molecules (see chapter 1.2.5). In this chapter the nature of the IgM subset shall be described in more detail.

Spontaneously secreted IgM can be found in all mice, even in naïve mice and those that are raised germ free or antigen free. This IgM is so called natural IgM (nIgM) and it is thought that it is produced by B-1a cells (Baumgarth et al., 2005; Kawahara et al., 2003; Thurnheer et al., 2003). Natural antibodies are important for the first line of defense against pathogenic bacteria (Zhou et al., 2007) as *Streptococcus pneumonia* (Haas et al., 2005) or pathogenic viruses (Baumgarth et al., 2000; Choi and Baumgarth, 2008) by guiding functional polarization of T cell responses. This is performed by promoting the recognition of pathogens by DCs or inducing class-switch recombination in activated B cells (see chapter 1.2.7) and inducing long-term immune memory (Fernandez Gonzalez S, 2008; Rapaka et al., 2010).

1.3.1 Membrane bound or secretory IgM

Membrane-bound IgM (mIgM) differs from its secretory form (sIgM). mIgM is a monomeric molecule while sIgM can be found in serum in pentameric or hexameric form (Kaveri et al., 2012; Sitia et al., 1990). The different appearance of mIgM and sIgM is due to a difference in the heavy chain (μ_H). The constant region of μ_H consists of four domains ($C_\mu1$-$C_\mu4$). (Rogers et al., 1980). In addition, the membrane μ_H consists of a hydrophobic C terminal sequence of 41 residues that can span the membrane. In contrast, the secretory μ_H consists of 4 constant domains and a hydrophilic C terminal sequence of 20 residues, (Rogers et al., 1980). Switching from membrane μ to secretory μ is achieved by alternative splicing of the primary mRNA.

1.3.2 Pentameric or hexameric IgM and the J chain

As mentioned before, secretory IgM can be detected as pentameric or hexameric form (Kaveri et al., 2012; Sitia et al., 1990). In serum, pentameric IgM is found in much higher concentrations than hexamers (Brewer et al., 1994; Shukala, 2009). Pentameric IgM consists of five IgM monomers and is generally associated with a joning (J)-chain. The monomers are covalently associated via disulfide bonds. The hexameric isoform consists of six monomers joined by disulfide bonds and does not contain the J-chain (Figure 1.5).

The J-chain is a small protein that becomes exclusively incorporated into polymeric IgA (dimer) and pentameric IgM. It is about 15 kDa and is bound via disulfide bonds to the terminal cysteine of heavy chains (Randall et al., 1992).

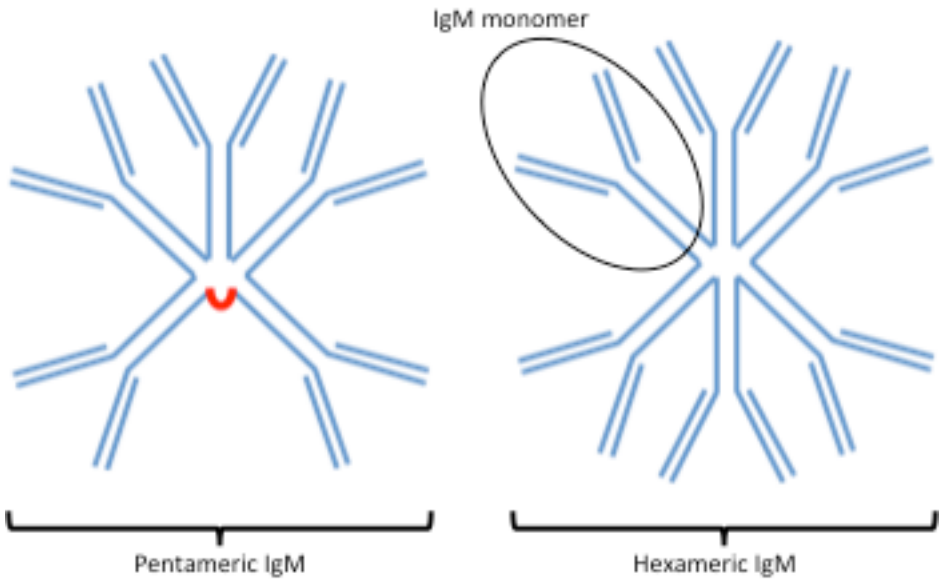

Figure 1.5 Schematic representation of pentameric and hexameric IgM.

Shown are the pentameric (left) and hexameric (right) isoforms of secreted IgM molecules. The pentameric IgM consists of five monomeric IgM molecules and is associated to a J-chain (red). The hexameric IgM consists of six monomeric IgM molecules without J-chain association.

When comparing hexameric and pentameric IgM, the hexameric isoform is more efficient in activating the complement system compared to pentameric IgM (Brewer et al., 1994; Davis et al., 1988). It is thought that T cell-dependent

immune responses lead to the stimulation of J-chain expression and therefore to a secretion of pentameric IgM, while T-independent immune responses might trigger the secretion of hexameric IgM (Brewer et al., 1994). The secretion of hexameric IgM might also be triggered preferentially when an enhanced complement activity is required, for example for elimination of invading pathogens. On the other hand, a higher activation of the complement cascade could lead to an overwhelming immune response if the secretion is not controlled. It is still not clear yet, which cells are secreting hexameric IgM.

1.4 Aim of the work

As described above, in mammals, B cell development starts already in the embryo thus a continuous production of B cells takes place. In the present work a mouse model was used in which B cell development can be switched on at any given time points such that the start of B cell development is synchronized. This mouse, called Indu-Rag1 contains an inverted and inverted floxed recombinantion-activating gene 1 (Rag1). Uninduced, it is lacking mature lymphocytes. These mice were crossed with MerCreMer mice, in which the Cre recombinase is fused to a mutated estradiol receptor and is under the control of the mb-1 promoter. In such mice (B-Indu-Rag1), Rag1 is not expressed but the expression can be induced in proB cells by oral administration of an estradiol analog, Tamoxifen (Düber et al., 2009). Using these transgenic mice, the developmental kinetics of B cell subsets and the usage of different V_H genes early during ontogeny can be studied. Furthermore, it could be analysed whether B-1a cells that develop newly in BM are contributing to the B1 cell pool of an adult mouse. In addition, the question arose which cells are producing hexameric IgM that is found in serum of mice and humans (Figure 3.29, (Kaveri et al., 2012; Shukala, 2009; Sitia et al., 1990)).

2 Materials and methods

2.1 Mice

B-Indu-Rag1 mice (Düber et al., 2009), CB20 and Rag1$^{-/-}$ mice were kept under specific pathogen free conditions (SPF) and maintained in the animal facility of the Helmholtz Centre of Infection Research. BALB/c mice were bought from Janvier. Mice aged between 8-14 weeks were used for the experiments. For induction of B cell development or as control, 400 µl of a 20 mg/ml solution of Tamoxifen (Ratiopharm) in ClinOleic (Baxter) was administered orally.

2.2 Flow cytometry and cell sorting

Single cell suspensions: Cells were obtained by flushing lymphoid organs or the peritoneum of mice with ice-cold IMDM (Iscove's modified Dulbecco's medium, GibcoBRL/Invitrogen). Erythrocytes in splenic, or bone marrow preparations were lysed by incubation with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 2-3 min on ice.

Preparation of blood cells: For the isolation of cells originating from blood, cardiac-blood was collected into 500 µl of PBS including 50 µl 1:100 diluted heparin 25000 (ratiopharm). Erythrocytes were lysed by adding Erythrocyte-lysis buffer (2.06 g Tris-base, 7.49 g NH_5Cl in a total volume of 1 l H_2O, pH 7.2) for 5 min at RT and afterwards centrifugation at 4 ˚C, 5 min at 1000 rpm was carried out. The supernatant was discarded and the steps were repeated until the cell pellet was white.

Extracellular staining: Cells were stained for 10-15 minutes on ice using antibodies coupled to FITC, PE, APC, APC-Cy7, PE-Cy7 or Biotin from the list below (Table 1.1). Samples were washed twice with FACS buffer (PBS with 2 % FCS and 0.5 M EDTA) afterwards. Biotinylated antibodies were counterstained in a second step with streptavidin-FITC (eBioscience), streptavidin-PE (Southern Biotech) or streptavidin-PerCPCy5.5 conjugates (eBioscience) for 10 min on ice, followed by a washing step with FACS buffer.

Table 2.1 Antibodies for FACS analysis

Antigen	Fluorescence	Clone	Company
Mac-1 (CD11b)	PE-Cy7	M1/70	eBioscience
CD3	FITC	145-2C11	eBioscience
CD5	PE	53-7.3	eBioscience
	APC		
CD4	APC	RM4-5	eBioscience
CD8	PE	53-6.7	eBioscience
CD25	PE-Cy7	PC61.5	eBioscience
B220	APC-Cy7	RA3-6B2	BDPharmingen
IgD	FITC	1.19	self made
CD19	APC-Cy7	1D3	BDPharmingen
	APC		
CD43	Bio	S7	BDPharmingen
c-kit	Bio	ACK-4	self made
CD21	Bio	7G6	BDPharmingen
IgM[a]	FITC	DS-1	BDPharmingen
	PE		
IgM[b]	PE	AF6-78	BDBioscience
CD40	PE	1C10	eBioscience
IgA	FITC	polyclonal	CaltayLaboratories
CD23	PE-Cy7	B3B4	eBioscience
AA4.1 (CD93)	APC	AA4.1	eBioscience

Intracellular staining for BrdU: Stainings were done according to manufacturer's protocol (FITC BrdU Flow Kit, BD Pharmingen).

FlowCytomix for immune globulin measurement: The staining and analysis of the immune globulin concentrations in serum samples were performed according to manufacturer's protocol (Mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Multiplex, eBioscience).

Flow cytometry was carried out using analyzers LSRII or Fortessa (Becton Dickinson). Data were analyzed using DIVA software (Becton Dickinson) or FlowJo. Cell sorting was carried out on a FACSAria (Becton Dickinson).

2.3 Adoptive cell transfer

$3*10^6$ splenocytes, isolated from Igα$^{-/-}$ mice (10-14 weeks old) were injected i.v. in a volume of 100-200 µl sterile PBS into B-Indu-Rag1, wt or Rag$^{-/-}$ mice and were analysed by flow cytometry to confirm the composition (40-50 % of T cells). Alternatively, splenocytes from BALB/c mice were negatively sorted for T cells according to manufacturer's protocol (Dynabeads® Untouched™ Mouse T Cells, Invitrogen) and injected at the same numbers as T cells from Igα$^{-/-}$ mice (10^6 T cells per mouse transferred).

2.4 Bone marrow transfer

8-12 weeks old B-Indu-Rag1 mice were used as donors. Bone marrow cells of both femurs were prepared and $3*10^6$ of these cells were injected i.v. into sublethally irradiated 8 weeks old Rag1$^{-/-}$ or CB20 mice. The recipients were induced 6 weeks after irradiation and analysed 3 weeks after.

2.5 ELISA

For collection of sera, blood was collected into Microvette 500 serum gel (Sarstedt, Germany). The tubes were kept at RT for 5 min and centrifuged for 5 min at 10000 g. The supernatants were taken for serum analysis and stored at -20 °C.

For collection of intestinal washout the small intestine was washed with 500 µl intestinal wash buffer (PBS including 0.1 mg/ml Trypsin-inhibitor, 50 mM EDTA, 0.1 % BSA). The flow through was collected into 1.5 ml tubes and centrifuged 10 min for 1000 rpm at RT. The supernatants were taken for intestinal washout samples and stored at -80 °C.

For ELISA MaxiSorb plates (MaxiSorb TM Immunoplates, Nunc) were coated over night with 1 µg/ml anti IgM (clone II/41, BD Pharmingen), goat anti-mouse IgG (Sigma) or 5 µg/ml goat anti-mouse IgA (Sigma) antibodies diluted in coating buffer at 4 °C. After washing six times with ELISA wash buffer (PBS,

0.05 % Tween-20), wells were blocked with 200 µl blocking buffer (PBS, 0.05 % Tween-20, 3 % BSA) for one hour at RT. Afterwards plates were flicked to discard the fluid. The intestinal washouts or sera from different mice were diluted in blocking buffer and added to the plate for 2 h at RT. After washing six times, 0.5 µ/ml biotinylated anti IgM (clone LO-MM-9, AbDSeroTec), peroxidase coupled goat anti-mouse IgG (Jackson ImmunoResearch), or peroxidase coupled goat anti-mouse IgA (Sigma) antibodies in dilution buffer (1 % BSA in ELISA wash buffer) were added and incubated for 1 h at RT. Plates were washed and if biotinylated antibody was added the previous step horseradish peroxidase (HRP) conjugated streptavidin (BD Pharmingen) in dilution buffer was dispensed into the wells. Plates were incubated for 45 min at room temperature and washed. Then the ELISA was developed at RT for 5-20 min in the dark adding 1 mg/ml o-Phenylendiamin (OPD) in substrate solution and 1:1000 diluted H_2O_2. The reaction was stopped adding 1 M H_2SO_4 and the results were read using an ELISA-reader (Dynatech, Germany) at a wavelength of 490 nm with reference wavelength of 340 nm.

2.6 ELISPOT

For ELISPOT MultiScreen IP plates (Millipore) were activated by adding 10 µl/well EtOH and washed with PBS. Afterwards the plates were coated either with 100 µl/well anti-IgM (clone II/41, BD Pharmingen), anti IgA (clone C10-3, BD Pharmingen) or goat anti-mouse IgG (Sigma) in sterile PBS. Plates were incubated overnight at 4 °C.

After washing once with blocking buffer (3 % BSA in PBS), wells were blocked with 100 µl blocking buffer for two hours at 37 °C. Afterwards plates were flicked to discard the fluid and washed with 200 µl PBS. The cell suspensions from different organs were serially diluted in IMDM medium and added to the plate for a overnight incubation at 37 °C 5 % CO_2 and 95 % humidity. The next day, after washing plates five times with washing buffer (0.01 % Tween-20 in PBS), 0.5 µ/ml biotinylated anti-IgM (clone LO-MM-9, AbDSerotec), biotinylated anti-IgA (clone 11-44-2, eBioscience) or biotinylated goat anti-mouse IgG (MABTECH) antibodies diluted in 0.01 % Tween-20 in blocking buffer were added and incubated for 30 min at room temperature. Plates were washed with 0.01 %

Tween-20 in blocking buffer and horseradish peroxidase (HRP) conjugated streptavidin (Pharmingen) in PBS was distributed into the wells. After washing and removing of Tween, plates were developed by adding 100 µl of substrate solution (333.3 µl of 10 mg AEC / ml DMF in 10 ml 0.1 M acetate solution with 5 µl H_2O_2). Plates were kept in dark for a few minutes until spots were observed. Reaction was stopped by washing plates under running water and removing the plastic sheath underneath. Plates were left to dry overnight in the dark. Results were read using an ELIspot-reader (BD biosciences)

2.7 RNA isolation

RNA isolation was done from sorted cell subsets either by purification with an RNeasy MiniKit according to the manufacturer's protocol, including DNase treatment on the column (RNeasy MiniKit, Qiagen) or isolated using peqGOLD TriFast according to the manufacturer's protocol (peqGold TriFast, qeqlab). The RNA concentrations were measured with a NanoDrop Spectrophotometer (peqLab) and samples were either further processed or stored at -80 °C.

2.8 cDNA preparation (RT-PCR)

Depending on the protocol for RNA isolation the RNA was transcribed into cDNA. After isolation using the RNeasy MiniKit no DNase treatment was necessary. 2 µg of RNA (max. 12 µl) were mixed with 1 µl of oligo $(dT)_{18}$ (Fermentas) on ice and incubated at 65 °C for 5 min. Afterwards 8 µl of a mastermix (4 µl 5x reaction buffer, 1 µl RiboLock RNase Inhibitor, 2 µl 10 mM dNTPmix, 1 µl Reverse Transcriptase or DEPEC H_2O as control, Fermentas) were added. The transcription was performed in a thermo cycler (5 min 25 °C, 60 min 42 °C, 5 min 70 °C). Finally the samples were taken up in the required amount of DEPEC water. The DNA concentrations were measured with a NanoDrop Spectrophotometer (peqLab) and samples were either further processed or stored at -20 °C.

If RNA was isolated using peqGold TriFast the DNA had to be digested first. This was done by adding 4 µl 5x Reaction buffer and 1 µl DNase (10 µg/µl Quiagen) to 2 µg RNA or a maximum of 15 µl of the RNA solution. The samples

were kept for 15 min at 37 °C following 10 min 60 °C incubation. Samples were then processed as described above.

2.9 PCR

2.9.1 Primer

Table 2.2 Primer sequences for PCR and qPCR

Gene	Primer name	Primer sequence
Secretor. IgM	forward	5'-TGTGTGTACTGTGACTCACAGGGA -3'
	reverse	5'-AGGGAGACATTGTACAGTGTGGGT-3'
Membr. IgM	forward	5'-TGTGTGTACTGTGACTCACAGGGA-3'
	reverse	5'-TGTAGAAGAGGCTCAGGAGGAAGA-3'
J-chain	forward	5'-CCTCTAGGATCATCCCTTCCACC-3'
	reverse	5'-AATGGTATACAAAGTTCCTTCTC-3'
RPS9	qPCRforRPS9	5'-TTGACGCTAGACGAGAAGGAT-3'
	qPCRrevRPS9	5'-AATCCAGCTTCATCTTGCCCT-3'
PAX-5	forward	5'-GCTACTCTGCACCGACGCTG-3'
	reverse	5'-GGGCTGCAGGGCTGTAATAGT-3'
BLIMP-1	forward	5'-AAGAGGTTATTGGCGTGGTAAG-3'
	reverse	5'-ACTTCCTGTTGGCATTCTTGG-3'
CD138	forward	5'-CCCCTCCTTTGACTTCTGCCT -3'
	reverse	5'-GCAGTCGGGTCCCCTTTCT-3'
XBP-1	forward	5'-TAGAAAATCAGCTTTTACGGGAGAAA-3'
	reverse	5'-GGGCCTGCACCTGCTGCGGACTCAG-3'
IgM	Cμ1	5'-ATGGCCACCAGATTCTTATCAGA-3'
	V_H cons.	5'-GAGGTGCAGCTGCAGGAGTCTGG-3'

2.9.2 Gel electrophoresis

For separation of the cDNA an agarose gel electrophoresis was performed. Therefore 2 % agarose (Appligene, Germany) were solved in 1x TAE (50 x TAE: 246 g Tris, 57.1 ml acetic acid, 100 ml 0.5 M EDTA, pH 8.0 in H_2O with a total volume of 1 l) and after heating 0.01 % DNA stain G (1:200000 in TAE, Serva)

were added for staining of the DNA. Gels were run at 120 V for 40 min and DNA was visualised with UV light (Transilluminator TI 2: Biometra, Germany) and documented. To analyse the size of the DNA-fragments, a marker was used (O'GeneRuler 100bp RNA Ladder Plus, Fermentas).

2.10 Real-time PCR (qPCR)

For quantitative real-time PCR cDNA samples were used undiluted or diluted and mixed with 10 µl of primer-mix (see Table 2.2), 10 µl of sample and 20 µl of CYBR green (Applied Biosystems). As control, a mixture of all samples for each gene of interest was serially diluted for comparison possibilities of the samples. The PCRs were done in a 7500 Real time PCR system (Applied Biosystems). The program was as follows:

50 ˚C	2'
95 ˚C	10'
40x	
95 ˚C	15"
Temperature depending on primer	45"
72 ˚C	2'
Dissociation step	
95 ˚C	15"
60 ˚C	1'
95 ˚C	15"
60 ˚C	15"

Temperatures for primers:

BLIMP-1	58 ˚C
CD138	58 ˚C
Secretory IgM	57 ˚C
XBP-1	61 ˚C
PAX-5	61 ˚C
Membrane IgM	55 ˚C
J-chain	55 ˚C
RPS9	54 – 62 ˚C

Samples were analysed with program 7500 (Applied Biosystems).

2.11 Sequence analysis

2.11.1 Preparation of cDNA

DNA was prepared as described above (see chapter 2.7, 2.8). Instead of oligo $(dT)_{18}$, 0.5 µl Cµ1 and 0.5 µl V_H cons (see Table 2.2) were used for amplification of IgH.

2.11.2 PCR

Afterwards a PCR was performed for amplification of IgH with specific primers added MIDs and Tags for identification of different samples after deep sequencing. Therefore primers of Table 2.3 were used. The reaction mix for the PCR were as follows (Thermo Scientific):

2 µl	TrueStart buffer
2 µl	NucMix 10mM
1 µl	V_H cons deep primer
1.6 µl	25 µM MgCl
0.25 µl	TrueStart HotTaq Polymerase (5 U/µl)
11.15 µl	DEPEC H_2O

18 µl of the reaction mix were provided into each tube and for each sample 1 µ of one of the Cµ2 deep primer and 1 µl of sample were added. PCR was performed according to following program on a thermo cycler peqStar (peqLab):

95 ˚C	1’	
95 ˚C	30”	
55 ˚C	40”	35 x
72 ˚C	40”	
72 ˚C	10”	

Table 2.3 Primer sequences for sequence analysis

Primer name	Primer sequence
Cmu2_deep1	5’-CTATGCGCCTTGCCAGCCCGCTCAGAGTCCATTTGGGAAGGACTGA-3’
Cmu2_deep2	5’-CTATGCGCCTTGCCAGCCCGCTCAGTAGGCATTTGGGAAGGACTGA-3’
Cmu2_deep3	5’-CTATGCGCCTTGCCAGCCCGCTCAGGCATCATTTGGGAAGGACTGA-3’
Cmu2_deep4	5’-CTATGCGCCTTGCCAGCCCGCTCAGCCGTCATTTGGGAAGGACTGA-3’

Cmu2_deep5	5'-CTATGCGCCTTGCCAGCCCGCTCAGAACTCATTTGGGAAGGACTGA-3'
Cmu2_deep6	5'-CTATGCGCCTTGCCAGCCCGCTCAGGTACCATTTGGGAAGGACTGA-3'
Cmu2_deep7	5'-CTATGCGCCTTGCCAGCCCGCTCAGCGTTCATTTGGGAAGGACTGA-3'
Cmu2_deep8	5'-CTATGCGCCTTGCCAGCCCGCTCAGGGTACATTTGGGAAGGACTGA-3'
Cmu2_deep9	5'-CTATGCGCCTTGCCAGCCCGCTCAGCACGCATTTGGGAAGGACTGA-3'
Vhcons_deep	5'-CGTATCGCCTCCCTCGCGCCATCAGGAGGTGCAGCTGCAGGAGTCTGG-3'

Gel electrophoresis was performed as described in chapter 2.9.2.

2.11.3 Purification of DNA from gels

For DNA purification from gels the MinElute Gel Extraction Kit (Qiagen) was used. Therefor the DNA bands of interest (approx. 400 bp) were cut out of the gel and the purification was done according to manufacture's protocol. DNA concentration was then measured with a NanoDrop Spectrophotometer (peqLab).

2.11.4 Sequencing

After purification of DNA from gels and measuring DNA concentration samples were passed to Irene Grünke (MHH), who kindly performed the sequencing and first analysis of data.

2.12 IgM purification

For IgM purification different strategies were followed (chapter 2.12.1 and 2.12.2 go together and chapter 2.12.3 to 2.12.5).

2.12.1 Radioactive labelling

Sort purified B cell subsets were incubated for 18 h with a mixture of DMEM without Cys or Met, 10 % IMDM, 3 % dialysed FCS, Pen/Strep (1:100) and 300 µCi/ 100 µl ^{35}S-Cys/Met (EasyTag^{TM} EXPRESS^{35}S Protein Labeling Mix, PerkinElmer).

2.12.2 Immunoprecipitation

Protein G immobilized agarose (Sigma) prepared according to manufacture's protocol. The supernatants were incubated with anti-IgM (clone II/41, BDPharmingen) for 2 hours at 4 ˚C. After this incubation Protein G beads were

added (25 µl to 500 µl supernatant) and incubated for 2 hours at 4 ˚C. After washing the beads with PBS, the beads were resuspended in 20 µl Glycin-HCl buffer (pH 2.7). After 15 min, the supernatant was taken into a new tube and neutralized with 0.1 N NaOH. Afterwards a native PAGE was run (see chapter 2.13).

2.12.3 Ammonium sulphate precipitation

Sera were mixed 1:1 with precipitation solution (1000 g/l ammonium sulphate in H_2O, pH 7) over night at 4 ˚C. Afterwards the mixture was centrifuged 10 min at 10000 rpm and the supernatant was discarded. The pellet was resuspended in PBS. Afterwards, the sample was dialysed at 4 ˚C over night in PBS and the samples were then used for IgM column (Econo-System) or stored at -20 ˚C.

2.12.4 Sepharose column for IgM purification

1 g of Sepharose (Sigma) was added into a 15 ml tube and washed four times with cold 1 mM HCl. In parallel 2 ml of coupling buffer (0.1 M NaHCO3, 0.5 M NaCl, pH 8.3) were added together with 2 mg of anti-IgM (clon M41.42, selfmade) in a dialyse tube and dialysed against coupling buffer. Afterwards 2 ml of the coupling buffer with M41.42 was added to the Sepharose and shaked overnight. The Sepharose was washed with 5 volumes of coupling buffer and blocked for two hours with 0.1 M Tris-HCl pH 8.0. The Sepharose was then washed repeatedly with 10 ml 50 mM Tris in 1 M NaCl pH 8.0 and 10 ml 50 mM Glycine in 1 M NaCl pH 3.5. Sepharose was packed into a column and washed with 50 ml of PBS. Serum was run through the column very slowly, after washing with PBS elution was achieved with Glycine buffer. The samples were collected and dialysed over night at 4 ˚C against PBS.

2.12.5 Concentration of IgM

Samples were loaded into a dialysis tube, placed into a tray and covered with Polyethylene glycol 40000 (PEG). It was until the volume in the tube was reduced to the required volume and sample was transferred into a new tube.

2.13 Native gel

The samples (either radioactively labelled or concentrated) were put onto a native PAGE agarose gradient gel (Novartis). As control, each time a

NativeMark Unstained Protein Standard (Invitrogen) was added into one lane. This marker contained also pentameric and hexameric IgM. Thus, it was possible to simply visualize both isoforms.

2.13.1 Development of gels with purified IgM from serum

Incubating the gel in 0.1 % coomassie solution R250 in 5 % acetic acid and 10 % Methanol and unstaining with H_2O was used to visualize the proteins.

2.13.2 Development of gels with radioactive labelled proteins

Fixation of gels was performed according to manufacture's protocol of the amplification reagent for increased detection efficiency (NAMP100, Amersham GE Healthcare). After keeping for 30 min in the amplification solution, gels were dried in a gel-drier (Biorad) for 30 min at 80 °C and exposed to an X-ray film (Amersham Hyperfilm MP, GE Healthcare) for several days. The film was then developed in a CURIX 60 (AGFA).

2.14 Statistical analysis

Statistical analysis was performed using Student *t* test with differences between means. Statistical *p*-values are given as * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.

3 Results

For the investigation of B cells development in adult mice the B-Indu-Rag1 mouse was used. With this mouse model the kinetics of newly developing B cell subsets and the usage of different V_H genes early during BCR recombination could be analysed. Furthermore the source of hexameric IgM should be discovered.

3.1 Inducible B cell development

The B-Indu-Rag1 mouse model was described before and proved that B cell development is inducible in such mice after Tamoxifen application. All B cell subsets could be found. In addition, it proved that BM derived progenitors in adult mice can develop into B-1a cells that populate the peritoneal cavity of such mice (Düber et al., 2009). These findings were obtained by several applications of the inducer before analysis of B cell subsets. Thus, synchronicity was lost to a large extend. Therefore, in the present work the induction was done only once to be able to analyse the differentiation steps of the newly developing B cells in a time dependent manner.

In the publication of Düber and colleagues it was shown that in uninduced B-Indu-Rag1 (InduRag mice on BALB/c background crossed to MerCreMer mice) mice an accumulation of $B220^+c\text{-}kit^+$ B cell progenitors could be detected similar to $Rag1^{-/-}$ mice (Düber et al., 2009). Upon induction of the B cell development these cells further developed and the percentage of the $c\text{-}kit^+$ B cells declines (Düber et al., 2009).

For answering the question how long it takes for a B cell pool to develop from $B220^+c\text{-}kit^+$ proB cells to a preB cell and to the subsequent populations, mice were induced once with Tamoxifen and analysed after five, six, seven, 11, 15 and 21 days. Non-transgenic littermates ($Rag1^{-/-}$ mice) were used as control mice that do not develop B cells upon Tamoxifen administration and BALB/c mice were used as positive wild type (wt) control.

3.1.1 Development of B cell progenitors in the bone marrow

In Rag1$^{-/-}$ mice an accumulation of B220^{+}c-kit^{+} proB cells in the BM can be detected (Düber et al., 2009) while in wt mice these proB cell differentiate into preB cells. PreB cells can be divided into large preB I and preB II and small preB II cell subsets. They can be detected by flow cytometric analysis using appropriate surface markers. For unknown technical reasons, preB I cells could not be analysed because the c-kit staining did not reveal a distinct population. In the preB II population, large preB II cells express the surface markers B220^{+}AA4.1^{+}CD25^{+}CD40^{-}IgM^{-}, while small preB II cells do express CD40 in addition (B220^{+}AA4.1^{+}CD25^{+}CD40^{+}IgM^{-}).

The gating strategy is shown in Figure 3.1. The percentages of precursor B cell populations were calculated and the differences over time are shown in Figure 3.2. A clear increase of progenitor cell development from day 5 up to day 7 for the large preB II cells and from day 5 up to day 11 for the small preB II cells could be detected. On the days 7 and 11 the respective subset reached maximum (3-4 % or 0.5 % respectively). Since B cell development was only induced once, the percentages of the pre B II cell subsets decline after reaching the peak as no further proB cell differentiation replenishes the preB cell compartment.

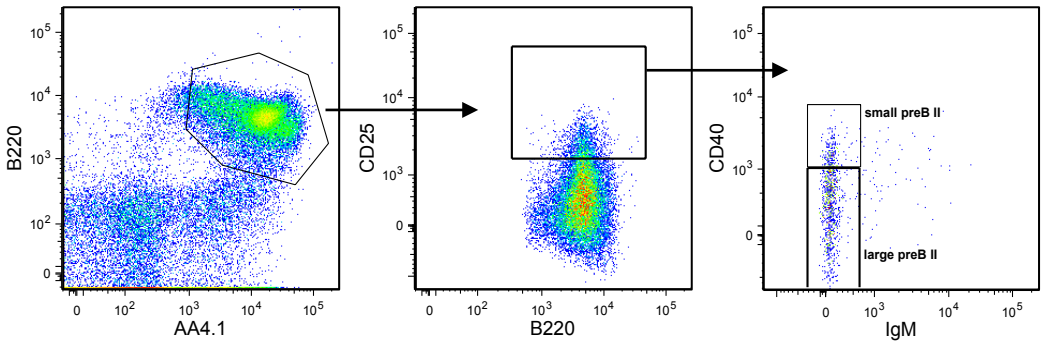

Figure 3.1 Flow cytometric gating for large (CD25^{+}CD40^{-}) and small (CD25^{+}CD40^{+}) preB cells.

Shown is an example staining for BM cells of a B-Indu-Rag1 mouse analysed six days after induction. Cells were gated for lymphocytes first by checking forward and sideward scatter. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Those cells were then gated for B220^{+}AA4.1^{+} cells for immature B cell population as shown. These cells were then gated for CD25 and CD40, IgM was excluded.

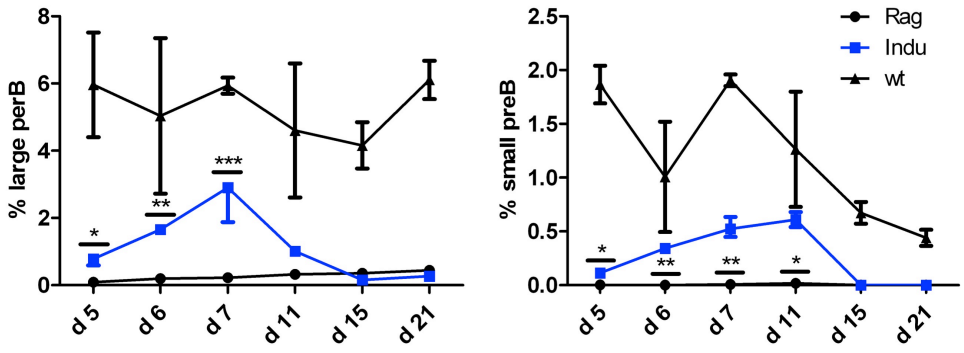

Figure 3.2 Development of B cell progenitors in bone marrow.

After induction of B cell development mice were analysed at indicated time points. Blue line represents induced mice (Indu), while black lines with circle represent the percentages of indicated progenitor population in Rag1$^{-/-}$ mice (Rag) and black line with triangles indicates the populations from wt mice. Stars represent significance between Indu and Rag mice.

3.1.1.1 Sequence analysis of early B cell progenitors

The V region in the murine heavy chain contains 15 different families and more then 100 different gene segments (Chevillard et al., 2002; Chowdhury and Sen, 2004). It was shown before that during fetal development of B cells the V gene segments proximal to the constant region are primarily used. Similarly, during early B cell development in adult BM such gene segments are found more often compared to their representation in the mature splenic B cell pool (Malynn et al., 1990).

Apparently, the pre-BCR plays an important role for the V_H selection in B cells during adulthood, while it inhibits clonal expansion of preB cells in fetal liver (Wasserman et al., 1998). Depending on the location of the particular V_H family, their activation for rearrangement is dependent on different signals e.g. gene segments, most distal of Cμ require IL-7 (Chowdhury and Sen, 2001). As consequence, a preferential usage of the proximal V_H gene segments early and rearrangement of distal gene segments later during ontogeny was suggested. However, thus far this was never directly shown.

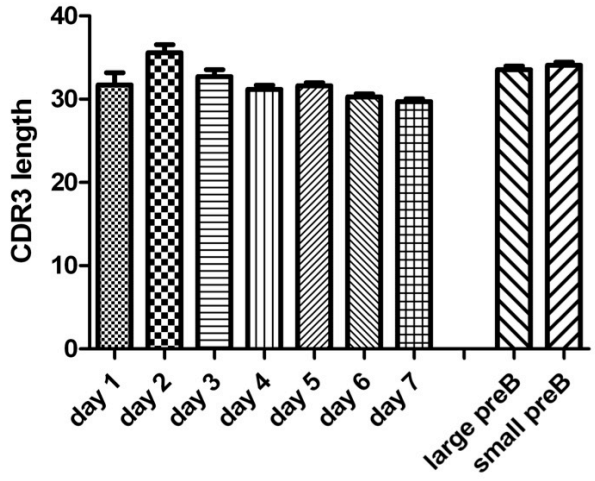

Figure 3.3 CDR3 length does not differ during early BCR rearrangement

BM cells were isolated at indicated time points after induction and CDR3 length for IgM heavy chain was analysed by deep sequencing. As control large and small preB cells were isolated from BALB/c mouse.

Thus, a time course of V_H usage was established from day 1 after induction up to day 7, which coincided with the peak of large pre-B cells (Figure 3.2). In addition, large and small preB cells, isolated from BALB/c BM cells were included in the analysis. This experimental setup made it possible to analyse the very early gene-usages during B cell development without including cells that might have undergone several steps of rearrangement. First, this experiment revealed that the length of CDR3 at different time points remains the same (Figure 3.3). More importantly, as predicted, the V_H usage varies over time (Figure 3.4). A clear shift from proximal to distal gene usage takes place. Although this result needs to be reproduced which was not possible for time constrains, it becomes clear that the postulated order of V_H gene segment usage could be confirmed.

3.1.2 The occurrence of transitional B cells in different organs peaks at day 11 post induction

Immature B cells migrate out of the bone marrow via the blood stream into the spleen where they further mature. These B cells can be classified according to their surface markers into transitional 1 (T1) and transitional 2 (T2) B cells. Both subsets are $CD19^+AA4.1^+$. Upon their expression of CD23 and IgM it is possible

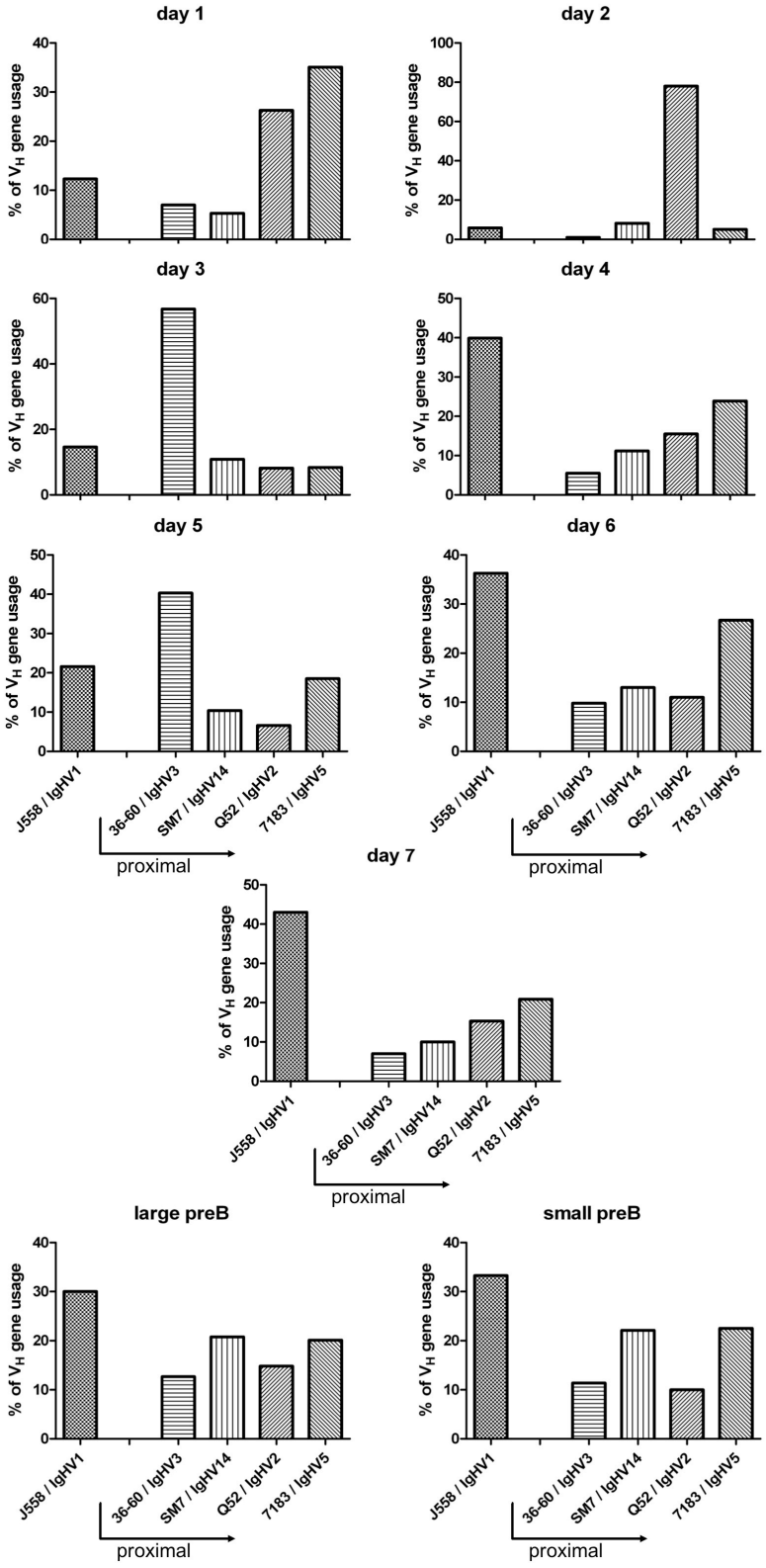

Figure 3.4 Proximal V_H genes are used first during early IgM rearrangement.

BM cells were isolated at indicated time points after induction and V_H family usage was analysed by deep sequencing. As control large and small preB cells were isolated from BALB/c mouse. Usage of four proximal (IgHV3, IgHV14, IgHV2 and IgHV5) and one distal (IgHV1) V genes are shown.

to distinguish these two populations (Allman et al., 2001). The gating strategy is shown in Figure 3.5.

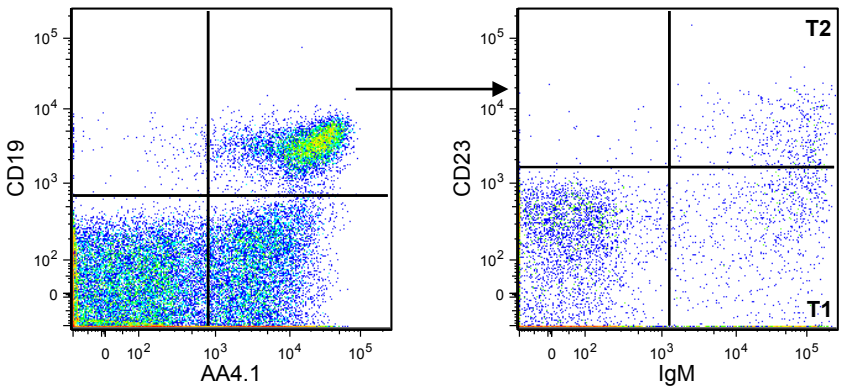

Figure 3.5 Flow cytometric gating for T1 and T2 cells.

Shown is a representative staining for cells isolated from BM of an induced Indu-B-Rag1 mouse. Cells were gated for lymphocytes first by checking forward and sideward scatter. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Those cells were then gated for $CD19^{+}AA4.1^{+}$ as B cell progenitors and upon CD23 and IgM expression T1 ($CD23^{-}IgM^{+}$) and T2 ($CD23^{+}IgM^{+}$) cells could be distinguished. Gating was performed for all analysed organs.

In Figure 3.6 the increase and decline of T1 and T2 cells after a single induction of B cell development are shown. In all analysed organs the highest percentage of transitional B cells could be found at day 11 after induction. A significant increase could already be detected seven days after induction for the T1 subset but not for T2 cells. This demonstrates that the cellularity first increased in the T1 and then in the T2 compartment. This also confirms the notion of a stepwise maturation of transitional B cells (Allman et al., 2001; Allman and Pillai, 2008). Since the T2 cells differentiate from T1 cells the T2 population starts to increase later compared to T1.

The sequential maturation also becomes very clear when the ration of the T1 and T2 compartments is compared. In adult wt mice the ratio between the two different transitional B cell subsets remains equal over time since a continuous generation and maturation takes place. As B cell maturation in B-Indu-Rag1 first starts with the time of induction, the ratio has to change over time. In Figure 3.7

the data for BM and spleen are shown. Interestingly, T1 cells disappear after 15 days, as the ratio declines nearly to zero in BM (Figure 3.7). At day 21 hardly any T1 or T2 cells could be detected in any organ of these mice. Thus, maturation is completed after this time period.

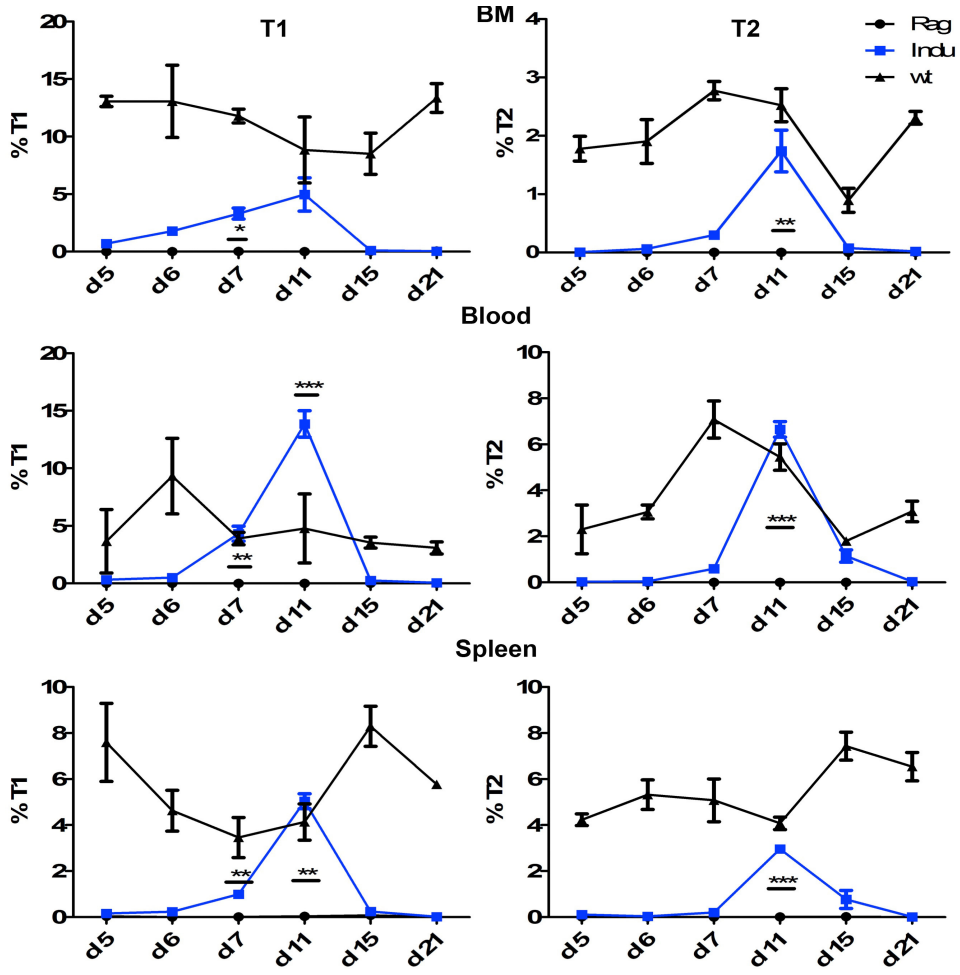

Figure 3.6 Development of transitional B cells in BM, blood and spleen.

After induction of B cell development, mice were analysed at indicated time points for the presence of T1 (left) and T2 (right) cells. The organs analysed were BM, blood and spleen (as indicated). Blue line represents induced mice, while black lines with circles represent the percentages of indicated transitional cells population in Rag1$^{-/-}$ mice, and black line with triangles indicate the populations isolated from wt mice. Stars represent significances between induced and Rag1$^{-/-}$ mice.

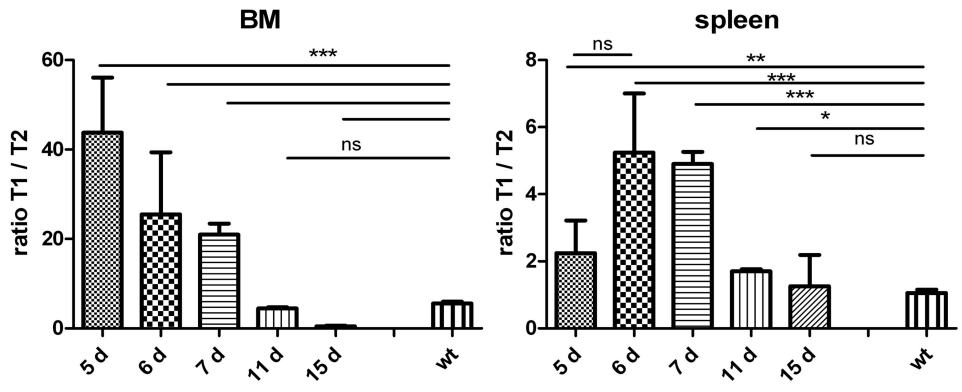

Figure 3.7 Ratio between T1 and T2 cells declines over time in induced mice.

The ratio of T1 to T2 cells was calculated by the percentage of these two subsets in BM (left) and spleen (right). Stars indicate significances.

3.1.3 Proliferation of transitional B cells in induced mice is similar to that of wt mice

Similar low levels of proliferation of T1 and T2 cells without BCR stimulation had been detected before (Petro et al., 2002). This suggested an expansion of both populations.

To study this idea in the B-Indu-Rag1 situation, the incorporation of BrdU by these cells was analysed. BrdU is an analogue of thymidine and can be incorporated into the newly synthesized DNA of replicating cells. Such cells can then be detected by using a BrdU-specific antibody. With the help of these antibodies the newly proliferating cells can be detected by flow cytometric analysis.

To this end, mice were induced once with Tamoxifen. As the highest percentage of transitional B cells could be detected at day 11 after induction (Figure 3.6), cells were analysed at day nine since the cells should be proliferative at that time. Therefore, nine days after induction BrdU was given i.p.. After two hours cells from bone marrow, spleen and blood were analysed. In blood, no proliferating transitional B cells could be detected but in BM and spleen some (2 % and 0.1 %) proliferating T1 cells could be found (Figure 3.8). No proliferation of T2 cells could be observed.

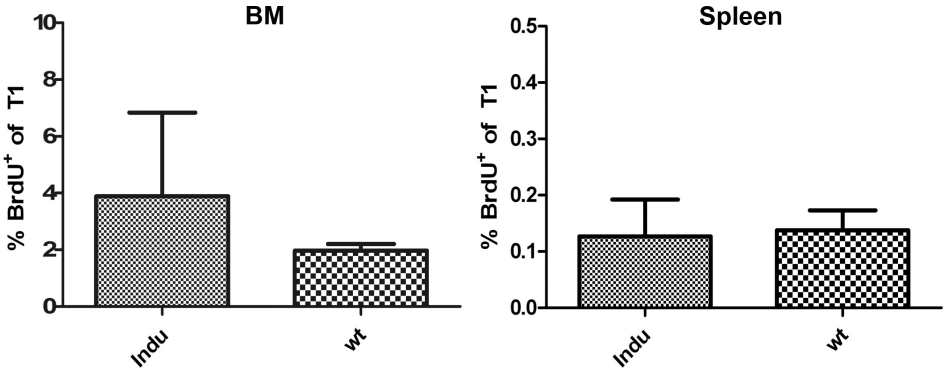

Figure 3.8 Proliferation of T1 cells in BM and spleen.

The proliferation of T1 in BM (left) and spleen (right) was analysed 9 days after induction. Therefore BrdU was given i.p. and two hours later the incorporation was analysed by flow cytometric analysis.

3.1.4 Mature B cell subsets in the spleen can be found from day 15 onwards

Immature B cells migrate from BM to the spleen and mature (Chung et al., 2003). To determine the time frame for this event, the first appearance of mature B cells was analysed. Mice were induced once and at different time points (day 5, 6, 7, 11, 15 and 21 after induction) appearance of the splenic B cell populations was established. All mature B cells are expressing CD19 and due to their differential expression of CD21, CD23 and CD5 they can be divided into the different subsets. An example of staining is shown in Figure 3.9.

All B cell subsets can be found in significant numbers at day 15 after induction (Figure 3.10). While the percentages of B-1a cells and MZ cells approach the percentages of wt mice, the percentage of B-2 cells remains relatively low compared to wt mice. This might be due to the self-renewing capacity of B-1a and MZ B cells. In general, the percentages of B-1a cells and MZ cells (induced mice 0.3 % or 3 %) are much lower compared to that of the B-2 cell population (induced up to 10 %) (Figure 3.10).

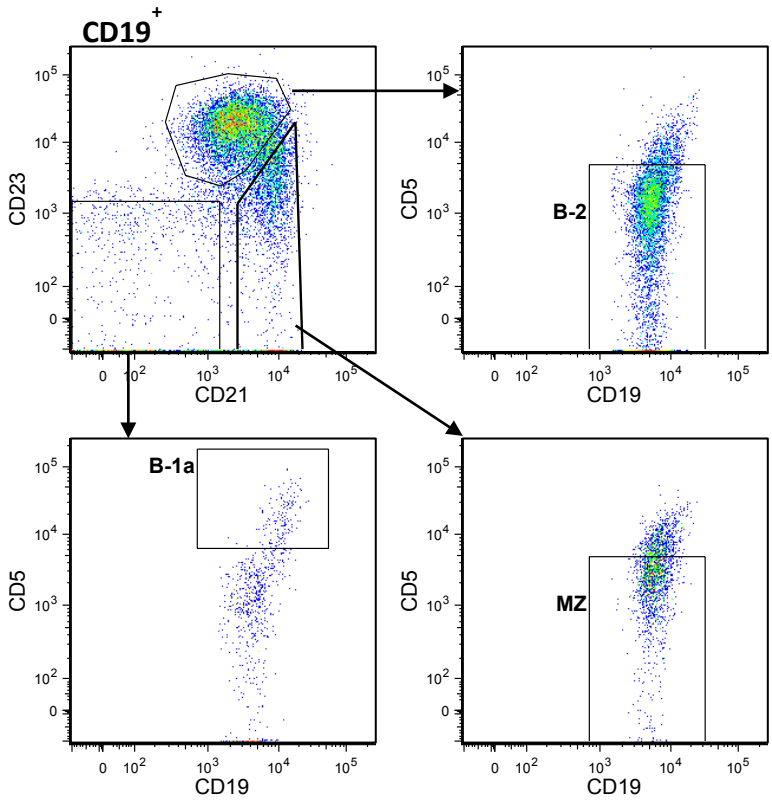

Figure 3.9 Gating strategy for mature B cells in spleen.

Shown is a representative staining for cells isolated from spleen of an induced B-Indu-Rag1 mouse. Cells were gated for lymphocytes first by checking forward and sideward scatter. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Those cells were then gated for CD19 and furthermore classified according to the figure and CD23, CD21, CD5 expression.

3.1.5 First mature B cell subsets in the bone marrow are detectable at day 15

Not all mature B cells are residing in the spleen. Some migrate to other organs and some, after maturation in the spleen, migrate back to the bone marrow (Allman and Pillai, 2008). The time for the first mature B cells to develop and migrate back to the BM was analysed. For this, B cells were divided into two populations: Both expressing CD19, one population, mainly B-2 cells are $IgM^{+}IgD^{+}$ and another, consisting of B-1a cells, are $IgM^{hi}IgD^{lo}$ (Figure 3.11). It is thought that especially $IgM^{+}IgD^{+}$ B cells in the BM is the main population that matures in the spleen and recirculates back to the BM (Loder et al., 1999).

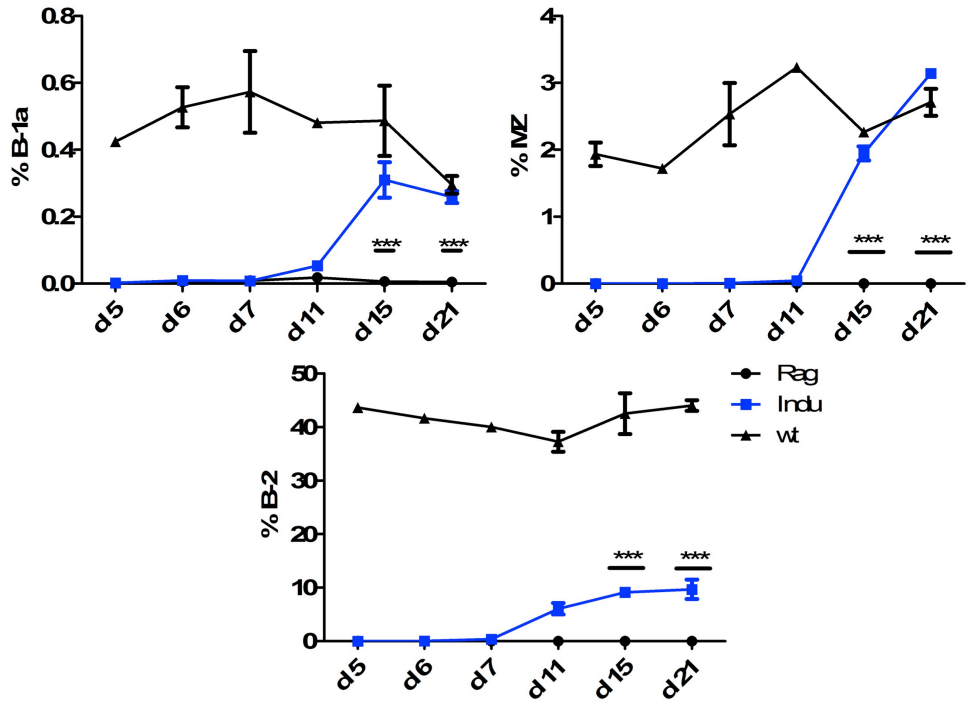

Figure 3.10 Percentages of mature B cell subsets in the spleen increase over time.

After induction of B cell development, splenocytes of mice were analysed at indicated time points for the presence of B-1a (upper left), MZ (upper right) and B-2 (low) cells. Blue line represents induced mice, while black lines with circles represent the percentages of indicated transitional cells population in Rag1$^{-/-}$ mice, and black line with triangles indicate the populations isolated from wt mice. Stars represent significances between induced and Rag1$^{-/-}$ mice.

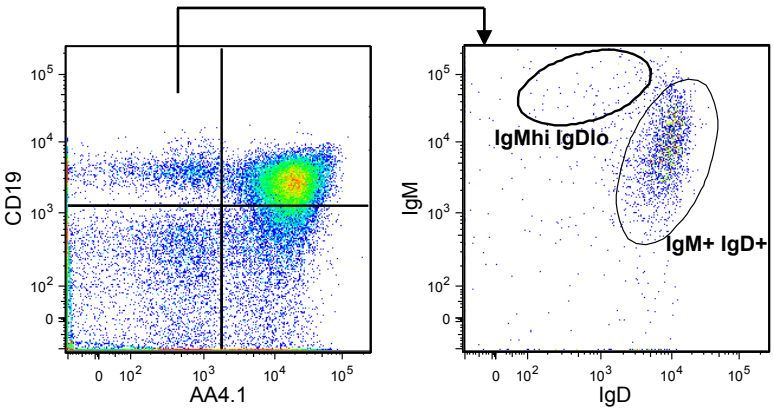

Figure 3.11 Gating strategy for mature B cells in BM

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Shown is a representative staining for cells isolated from BM of an induced B-Indu-Rag1 mouse. Cells were gated for lymphocytes first by checking forward and sideward scatter. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Immature B cells were excluded by gating for $CD19^+AA4.1^-$ cells that could be further divided into $IgM^{hi}IgD^{lo}$ and IgM^+IgD^+ cells.

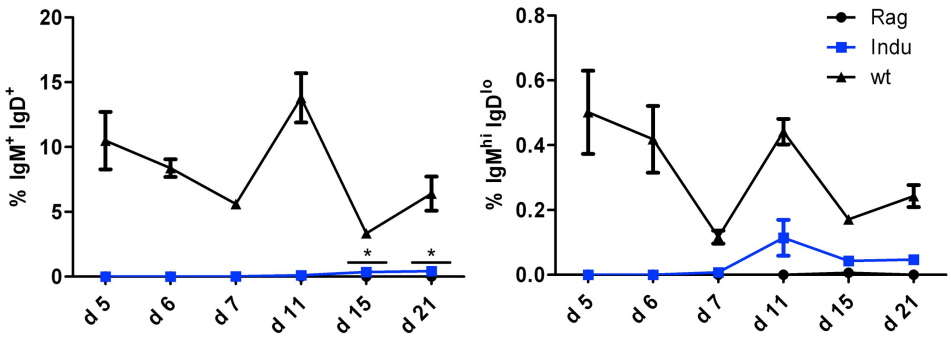

Figure 3.12 Increase of IgM^+IgD^+ cells in BM 15 days after induction

After induction of B cell development, BM cells were analysed at indicated time points for the presence of IgM^+IgD^+ (left) and $IgM^{hi}IgD^{lo}$ (right) B cells. Blue line represents the induced mice, while black lines with circles represent the percentages of indicated transitional cells population in $Rag1^{-/-}$ mice, and black line with triangles indicate the populations isolated from wt mice. Stars represent significances between induced and $Rag1^{-/-}$ mice.

The results showed that the $IgM^{hi}IgD^{lo}$ population of B1a cells does not increase significantly upon induction. However, 15 days post induction the first mature IgM^+IgD^+ cells can be detected in the BM (Figure 3.12). In general, like in wt mice, only very low percentages of $IgM^{hi}IgD^{lo}$ B cells could be detected in the BM (max. 0.6 %, Figure 3.12), while the main mature B cell population in the BM consists of IgM^+IgD^+ B cells. In induced mice, the IgM^+IgD^+ population rises up to 0.6 %, whereas it is about 7 % in wt mice (Figure 3.12).

3.1.6 Mature B cell subsets in the peritoneal cavity are found from day 11 onwards and can still be detected after 8 weeks

The peritoneal cavity is the site where B-1a cells mainly reside. These cells are the main producers of natural IgM. They can react to T cell-independent stimulation and act as innate-like cells (Alugupalli et al., 2004). Their task in the immune system is to quickly produce antibodies upon infection. In contrast to B-

2 cells, B-1a cells are self-renewing and their progenitors are mainly found in fetal liver. Nevertheless, also in the adult BM some progenitors for B-1a cells are present. Therefore newly developing B-1a cells might be found in the periphery (Düber et al., 2009). Two other B cell populations commonly found in the peritoneal cavity are B-1b and B-2 cells.

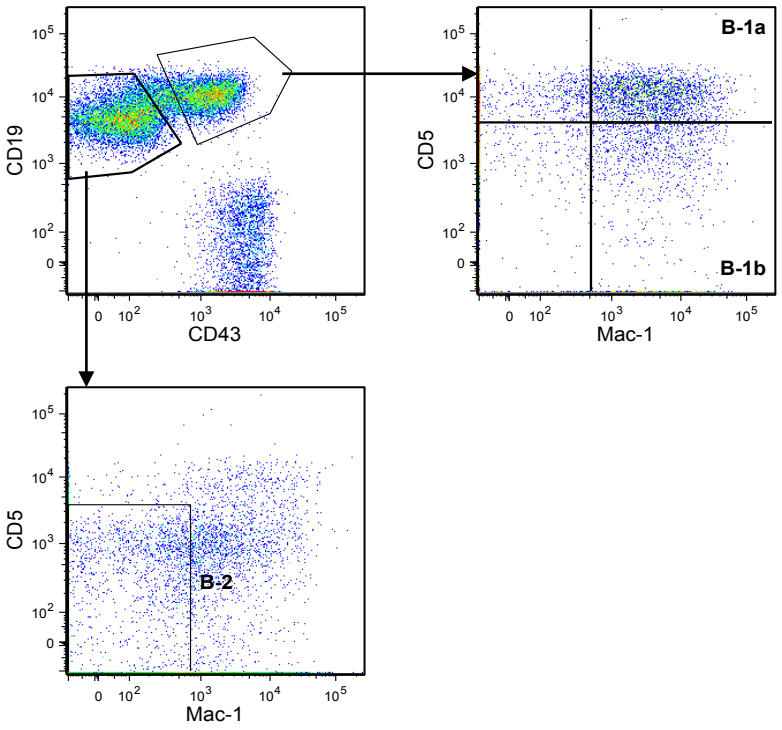

Figure 3.13 Gating strategy for peritoneal mature B cell populations

Shown is a representative staining for cells isolated from peritoneal cavity of an induced B-Indu-Rag1 mouse. Cells were gated for lymphocytes first by checking forward and sideward scatter. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Cells were further subdivided upon their expression of CD19, CD43, Mac-1 and CD5.

In Figure 3.13 an example of staining for the B cell subsets in peritoneal cavity is shown. While both B-1 cell subsets express CD19, CD43 and Mac-1, only B-1a cells are also expressing CD5 on their cell surface. B-2 cells on the other hand are expressing only CD19 and are negative for the other markers.

It could be shown that in the peritoneal cavity first B-2 cells can be detected 11 days after induction, whereas the B-1 cell subsets are found from day 15

onwards (Figure 3.14). While the percentages of B-1a and B-2 cells are not reaching the level of wt mice, the percentage of B-1b cells in the peritoneal cavity is rising above wt-level (17 % compared to 6 % after eight weeks, Figure 3.14). Also the absolute number of B-1b cells in induced mice is reaching that of wt mice eight weeks after induction. In contrast, the absolute numbers of the other subsets do not reach wt-level (data not shown).

3.1.7 No significant differences in B cell proliferation between induced and wt mice

To analyse whether the increase of the B cell subsets in B-Indu-Rag1 mice is due to proliferation of these cells during development or whether they exclusively differentiate from progenitors, the proliferation of $CD19^+$ B cells was analysed by injecting BrdU 15 days post induction. This time point was chosen as the highest numbers of the different subsets could be detected at day 21 after induction and day 15 was the first day all subsets could be seen in significant numbers (Figure 3.10, Figure 3.14). It could be shown, that there was no significant proliferation comparing $CD19^+$ cells isolated from spleen or peritoneal cavity from induced mice compared to wt mice (Figure 3.15).

Therefore the question remains where the higher percentages, especially of B-1b cell population over time arise from, as no progenitors can be detected after day 11 and the transitional B cell subsets also vanish at day 21 (Figure 3.15).

3.1.8 Immunoglobulin concentrations increase over time in serum and gut

To analyse the functionality of the induced B cell populations, it is necessary to investigate their ability to produce immunoglobulins and whether this is comparable to wt mice. Therefore, the concentration of immunoglobulins in serum of the different mouse groups was analysed using Mouse Immunoglobulin Isotyping Kit.

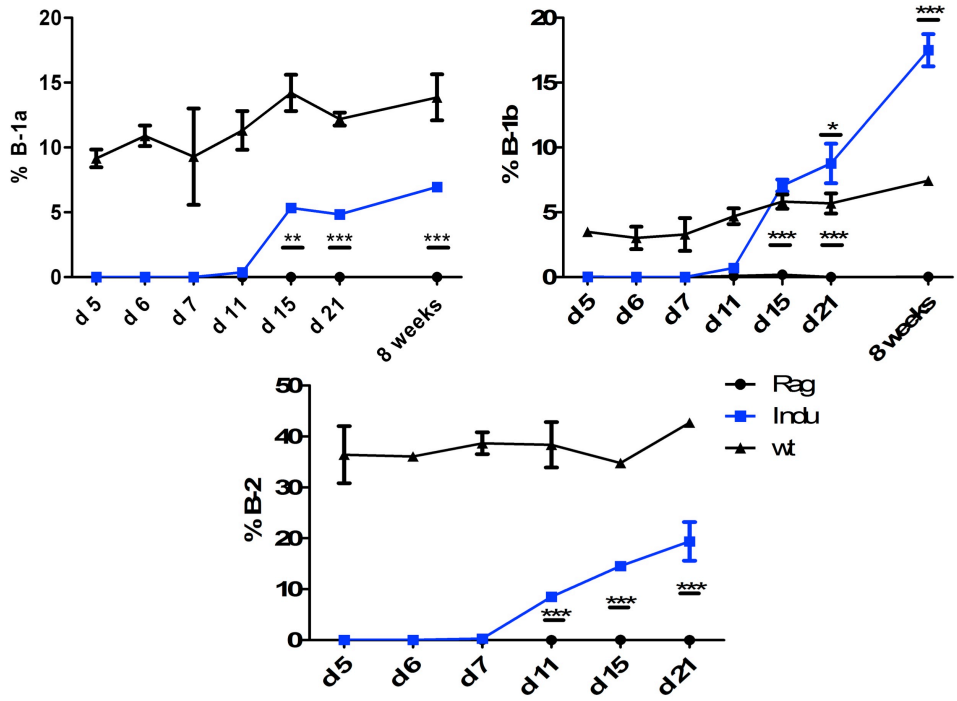

Figure 3.14 Mature B cell subsets in peritoneal cavity rise from day 11 or 15 onwards

After induction of B cell development, peritoneal cavity cells of mice were analysed at indicated time points for the presence of B-1a (upper left), B-1b (upper right) and B-2 (low) cells. Blue line represents induced mice, while black lines with circles represent the percentages of indicated transitional cells population in Rag1$^{-/-}$ mice, and black line with triangles indicate the populations isolated from wt mice. Stars represent significances between induced and Rag1$^{-/-}$ mice.

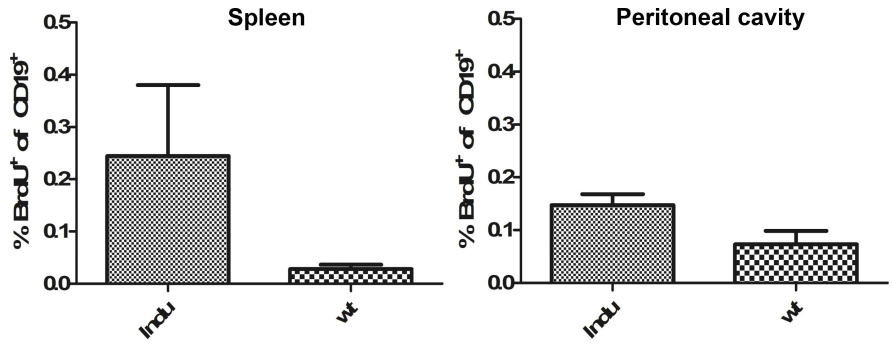

Figure 3.15 No significant differences in B cell proliferation between induced and wt mice.

The proliferation of CD19^{+} B cells isolated from spleen (left) or peritoneal cavity (right) was analysed 15 days after induction. Therefore BrdU was given i.p. and two hours later the incorporation was analysed by flow cytometric analysis.

With this experiment it was possible to show, that comparable to the increase of the B cell subsets in the different organs the first immunoglobulins could be detected at day 15 after induction (Figure 3.16). Nevertheless, this is true only for IgM, IgG2a and IgG3. The other immunoglobulin subsets can be detected first in significant amounts after 21 days of induction (IgA and igG2b), while IgG1 was not increasing significantly at all. At the same time, the highest concentration could be detected for IgM with a tendency of reaching wt levels. This is the only immunoglobulin that rises to a concentration of 50 µg/ml. All other immunglobulins do not rise above 1 µg/ml (Figure 3.16). Switching of B cells to one of the other immunoglobulins is obviously not supported since it might require T cell help.

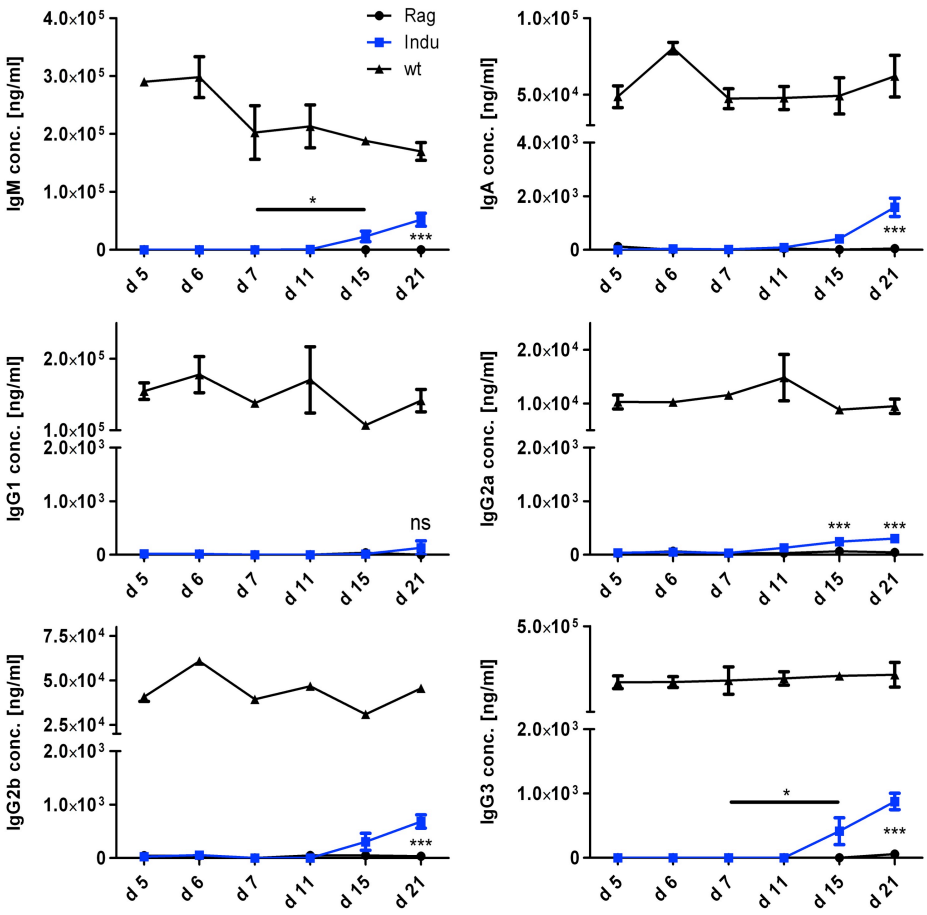

Figure 3.16 Immunoglobulin concentrations increase in serum of induced mice.

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The indicated immunoglobulin concentrations in serum of the indicated mouse groups were measured using Mouse Immunoglobulin Isotyping Kit. Days after induction are indicated. Blue line represents induced mice, while black lines with circles represent the percentages of indicated transitional cells population in Rag1$^{-/-}$ mice, and black line with triangles indicate the populations isolated from wt mice. Stars represent significances between induced and Rag1$^{-/-}$ mice.

In addition to their residence in the peritoneal cavity, B-1 cells are able to migrate to intestinal lamina propria (Allman and Pillai, 2008). For some time, it was believed that they are key players in the intestinal immune response by switching to IgA secreting cells in a T cell independent manner (Allman and Pillai, 2008). However, recently it was shown that IgA^{+} cells, isolated from intestine might not derive from B-1a, but rather from B-1b cells (Roy et al., 2013). To analyse the functionality in the induced mice and to investigate the time IgA requires to accumulate in the gut, intestinal washout was analysed by ELISA for IgA at 11, 15 and 21 days after induction.

In the induced mice, nearly no increase of IgA concentration in the intestinal wash out was detectable (Figure 3.17). The concentration does not increase further than 230 ng/ml at day 21 after induction, compared to 500 µg/ml in wt mice.

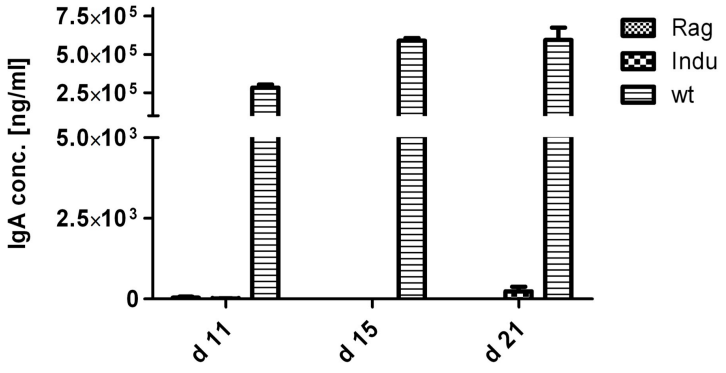

Figure 3.17 IgA concentration in intestinal wash out is not significantly increased after induction

IgA concentrations of the intestinal washout of different mouse groups (Rag1$^{-/-}$, induced mice and wt control) were measured by ELISA at indicated time points after induction.

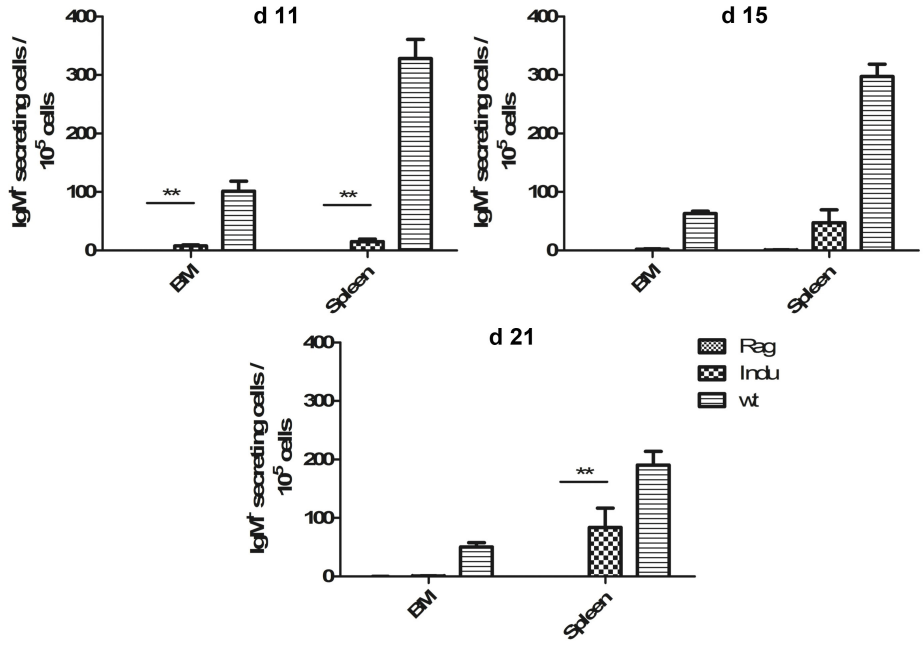

Figure 3.18 Amount of IgM secreting cells increases in spleen over time

Number of IgM secreting cells were measured by ELISPOT in BM and spleen at the indicated time points after induction from Rag1$^{-/-}$, induced and wt mice.

3.1.9 Increase of IgM secreting cells over time

To confirm the IgM concentration in serum of the induced mice is due to the increase of B cells that are secreting IgM and not due to a few B cells that secrete high amounts IgM over time, ELISPOTs for the different time points for spleen and BM were performed. The results are shown in Figure 3.18. The number of IgM secreting cells is increasing over time in the spleen. While there are only 15 IgM secreting cells/ 10^5 cells detectable in spleen 11 days after induction, this number increases to 125 IgM secreting cells/ 10^5 cells at day 21 after induction. In the BM only at day 11 very low numbers of IgM secreting cells could be detected in the induced mice suggesting that such IgM secreting cells are short lived plasma cells.

3.2 T cell requirement for B cell development

It is known that for the induction of B cell responses, especially B-2 cell activation, T cell help is required. Recently it was reported, that in humanized mice, T cells are also required for the maturation of human B cells as such cells would rest in the transitional stage without T cells (Lang et al., 2013). To analyse whether there is some influence of T cells on B cell maturation in mice, T cells were adoptively transferred one day before induction of B cell development. As it could be shown here that B cells can mature without T cells, the question whether increased B cells development would become apparent and whether there might be a difference in B cell composition. Thus, the mice were analysed 21 days after induction. At this time point all subsets were present as pointed out above. In addition, mice were analysed after eight weeks, to reveal whether there is a difference in long-term maintenance.

3.2.1 No differences when injecting T cells isolated from wt spleen or injection of splenocytes isolated from Igα$^{-/-}$ mice

For analysing the requirement of T cells for B cell development, splenocytes of Igα$^{-/-}$ mice (that do not have B cells) were injected intravenously into B-Indu-Rag1 mice.

As T cells from Igα$^{-/-}$ mice might harm B cell development (Roy, unpublished data), differences in B cell development using whole splenocytes from Igα$^{-/-}$ mice and negatively isolated T cells from BALB/c splenocytes should be tested. With the help of flow cytometric analysis the percentage of T cells in splenocytes of Igα$^{-/-}$ mice was calculated and the same number of T cells isolated from BALB/c splenocytes were injected into mice to ensure comparable numbers (Figure 3.19). One day after adoptive transfer of T cells, B cell development was induced and mice were analysed 21 days after induction. As shown in Figure 3.20, no differences between the B cell subsets in spleen or peritoneal cavity could be observed. Therefore splenocytes from Igα$^{-/-}$ mice did not harm the B cell development and could furthermore be used as T cell donors for the experiments.

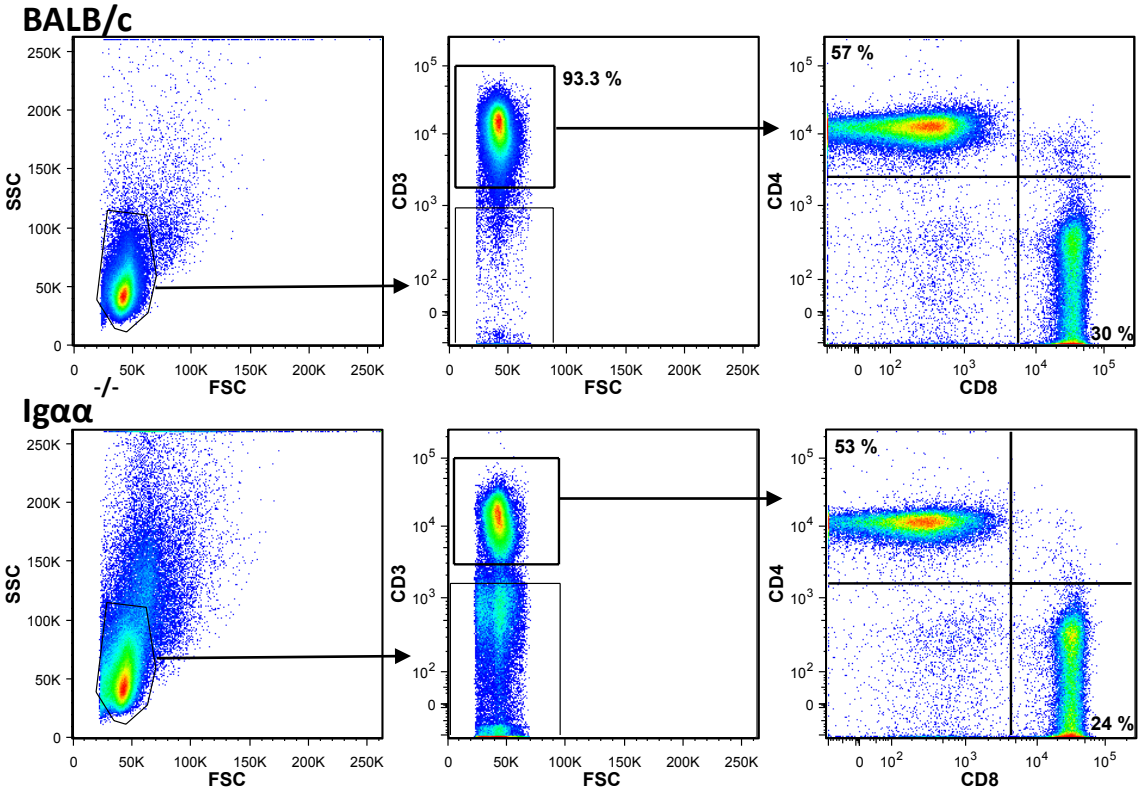

Figure 3.19 Transfer T cells from BALB/c or Igα$^{-/-}$ mice have same containing percentages of CD4^{+} and CD8^{+} T cells

Transfer T cells from BALB/c mice (upper panel) or Igα$^{-/-}$ mice (lower panel) were analysed for the appearance of CD4 or CD8 upon CD3^{+} T cells. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Number in quadrants in far right dot plots indicate percentages of subsequent T cell subsets from all T cells.

3.2.2 Number of mature B cells in spleen after induction and T cell administration is higher than without T cells

The results for spleen as the primary organ of maturation are shown first. In Figure 3.21 the different mouse groups are shown 3 and 8 weeks after induction. The B cell subsets could be divided into B-1a, MZ and B-2 cells (see chapter 3.1.4). For B-1a cells it could be shown, that this population is found in significantly higher numbers when T cells are available (Indu+T) than without T cells (Indu). For MZ and B-2 cells this is true for the later (8 weeks) or earlier (3 weeks) time point respectively (Figure 3.21).

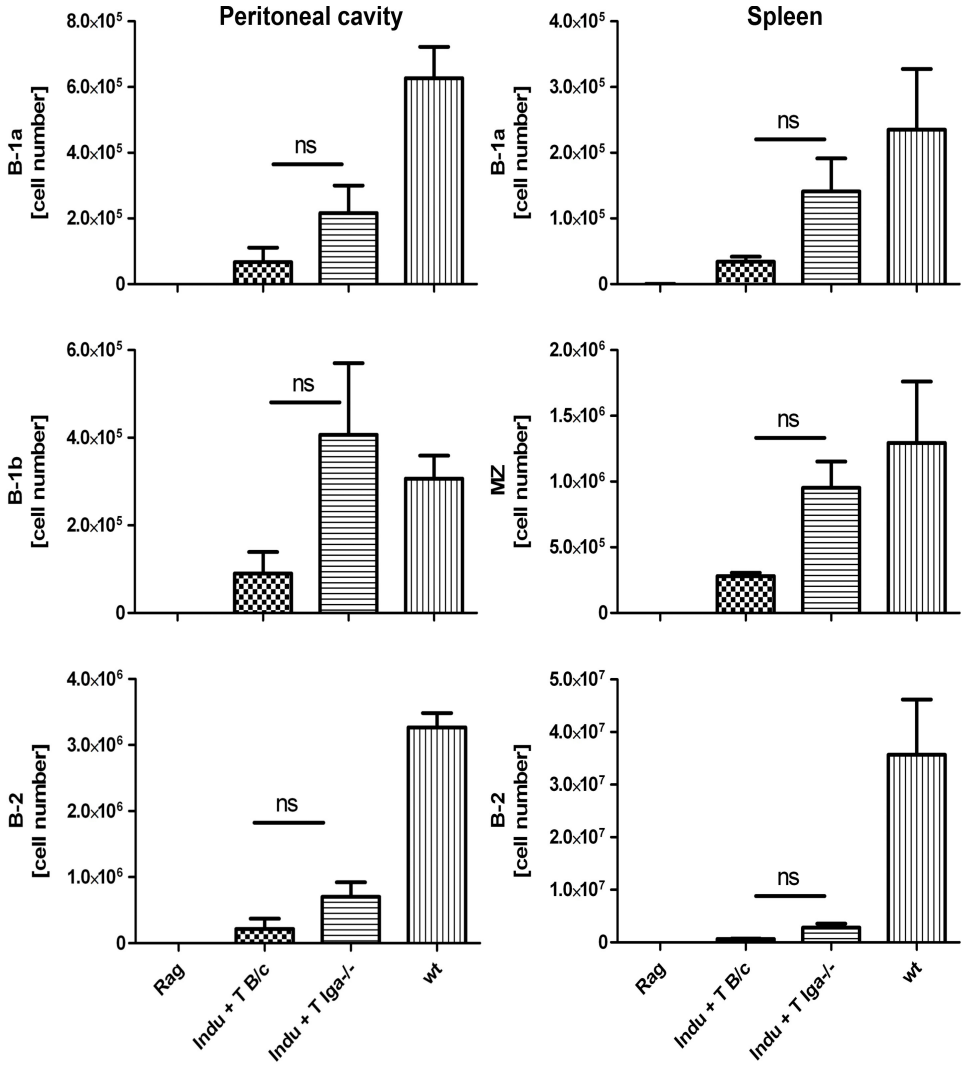

Figure 3.20 No significant differences when using T cells isolated from Igα$^{-/-}$ mice or BALB/c mice

Shown are total cell numbers for indicated B cell subsets in peritoneal cavity (left) and spleen (right). Control groups are Rag1$^{-/-}$ (Rag), and wt mice. For comparison induced mice that got T cells from BALB/c mice (Indu + T B/c) and induced mice that got T cells from Igα$^{-/-}$ mice (Indu + T Iga-/-) are shown.

3.2.3 Number of mature B cells in BM and peritoneal cavity after induction and T cell administration

For analysis of the B cell subsets in the periphery and of those cells that migrate back to the BM, the peritoneal cavity and the BM were analysed 3 and 8 weeks after induction. In the BM the mature B cells were divided into IgM^{+}IgD^{+} and IgM^{hi}IgD^{lo} B cells (see chapter 3.1.5). In the peritoneal cavity the different B cell

subsets B-1a, B-1b and B-2 could be distinguished by surface staining shown before (see chapter 3.1.6).

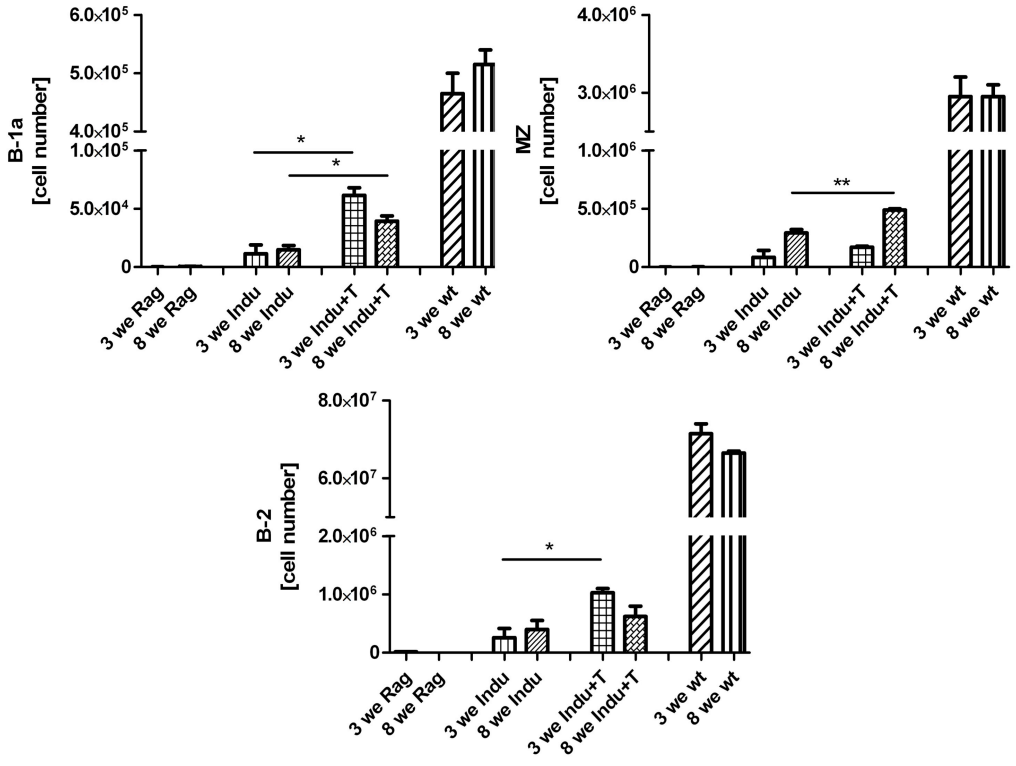

Figure 3.21 Increased number of B cells in spleen if T cells are available.

Indicated B cell subsets from different mouse groups are shown as indicated for 3 or 8 weeks after induction. Significances were calculated and marked by stars between induced mice (Indu) and induced mice additionally adoptive transferred T cells (Indu + T). Control groups are Rag1$^{-/-}$ (Rag) and wt mice.

In both organs nearly no significant difference in cell numbers could be observed whether T cells are available for their differentiation or not (Figure 3.22, Figure 3.23). In the BM nearly no mature B cells could be detected after induction, while in the peritoneal cavity cell numbers are increasing over time. Only in the B-2 cell subset a significant difference between Indu and Indu+T mice could be found three weeks after induction (Figure 3.23). At this time point more B-2 cell can be detected in those mice that had adoptively transferred T cells available. Five weeks later (8 weeks after induction) this significant difference could not be seen anymore (Figure 3.23). The cell numbers of all B

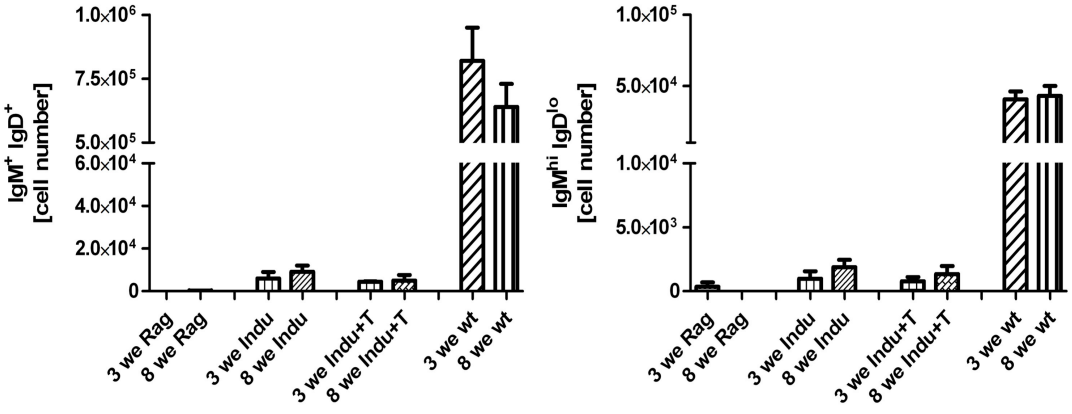

Figure 3.22 No significant difference between B cell subsets in BM whether T cells are available or not.

Indicated B cell subsets in different mouse groups are shown as indicated for 3 or 8 weeks after induction. Significances were calculated and marked by stars between induced mice (Indu) and induced mice additionally adoptive transferred T cells (Indu + T). Control groups are Rag1$^{-/-}$ (Rag) and wt mice.

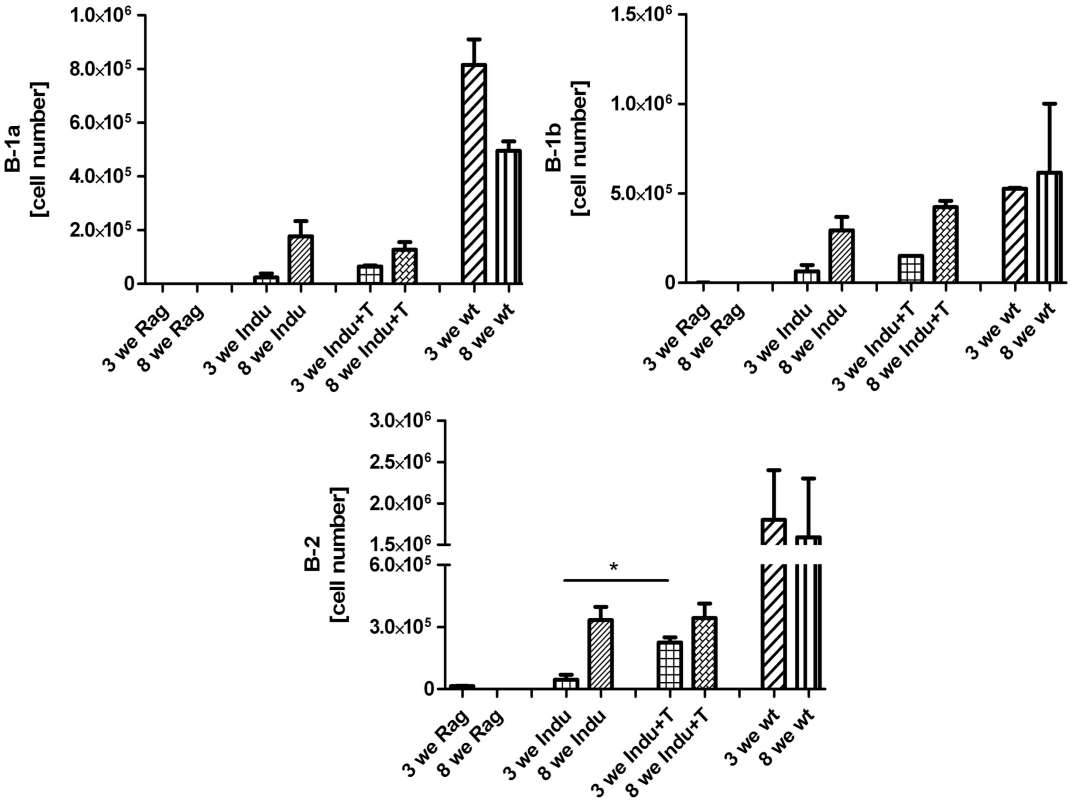

Figure 3.23 Only significant differences in B-2 cell population in peritoneal cavity whether T cells are available or not.

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Indicated B cell subsets in different mouse groups are shown as indicated for 3 or 8 weeks after induction. Significances were calculated and marked by stars between induced mice (Indu) and induced mice additionally adoptive transferred T cells (Indu + T). Control groups are Rag1$^{-/-}$ (Rag) and wt mice.

cell subsets, except B-1b cells in the peritoneal cavity, did not reach the cell numbers of those that could be seen for wt mice. The B-1b cell subset reached the level of wt mice 8 weeks after induction. This is in consensus with the higher percentage of B-1b cells after induction although no T cells were available (see chapter 3.1.6). There were no significant differences detectable with or without T cells available (Figure 3.23).

3.2.4 Immunoglobulin concentrations increase upon availability of T cells in serum and intestinal wash out

As T cells obviously influence the development of B cell maturation in some way, the effect on the immunoglobulin secretion was analysed. Therefor serum from mice was taken at different time points after induction and was analysed using Mouse Immunoglobulin Isotyping Kit. The results are shown in Figure 3.24.

No differencees in IgM concentrations between induced (Indu) and induced mice that have T cells available (Indu+T) could be observed. In contrast, for all other immunoglobulins (IgA, IgG1, IgG2a, IgG2b and IgG3) differences were found. For each immunoglobulin the concentration is significantly higher at day 21 post induction when T cells are available. Analysing later time points, no significant differences could be observed any more, except for IgA, there the significant higher concentration in mice that have seen T cells are also visible at 8 weeks post induction (Figure 3.24).

Similarly, in intestinal wash out the IgA concentration was altered. Mice that received adoptively transferred T cells show higher IgA concentrations compared to induced mice without T cell transfer after three and after eight weeks of induction (Figure 3.25).

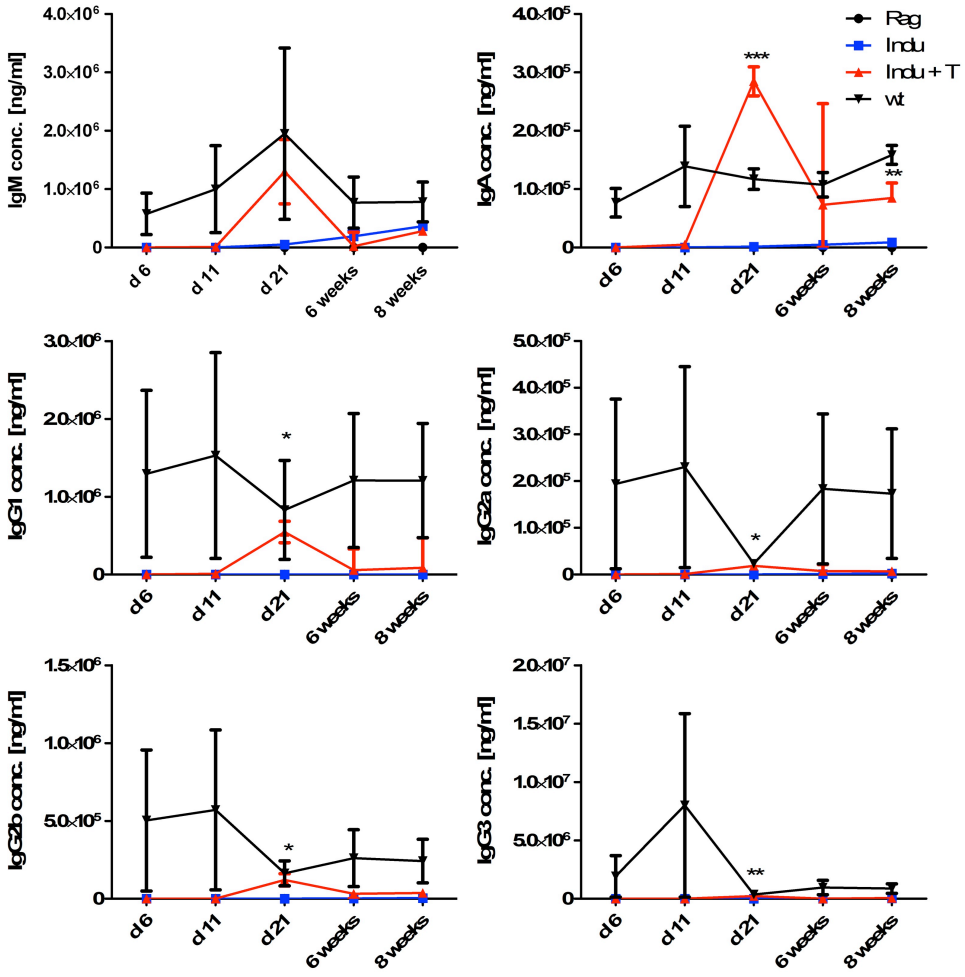

Figure 3.24 Immunoglobulin concentrations are slightly higher in induced mice if T cells are available.

The indicated immunoglobulin concentrations in serum of the indicated mouse groups were measured using Mouse Immunoglobulin Isotyping Kit. Days/weeks after induction are indicated. Blue line represents induced mice, red line represents induced mice that got T cell transferred, while black line with circles represent the serum concentration of indicated immunoglobulin in Rag1$^{-/-}$ mice, and black line with triangles indicate that of wt mice. Stars represent significances between induced and induced mice that recieved T cells (Indu+T).

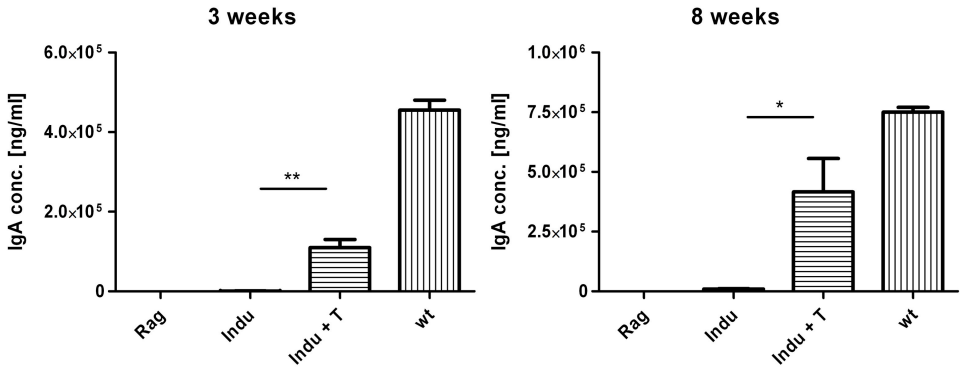

Figure 3.25 Higher IgA concentration in intestinal washout if T cells are available.

IgA concentrations of the intestinal washout of different mouse groups (Rag1$^{-/-}$, induced, induced+T mice and wt control) were measured by ELISA at three weeks (left) and eight weeks (right) after induction.

3.2.5 More immunoglobulin secreting cells when T cells are available

It is known that for the switching of naïve B cells to IgA, IgG and IgE T cells are required (Manis et al., 2002; Stavnezer et al., 2008). As the concentrations of immunoglobulins are much higher in those mice that had adoptively transferred T cells available (see chapter 3.2.4) the question arose whether this is due to a few B cells that secrete high numbers of immunoglobulins, or whether this is due to the fact that much more cells have switched from IgM to IgA or IgG - secreting cells. To analyse this, ELISPOTs for IgM, IgG and IgA were performed at day 11, 15 and 21 after induction for cells isolated from BM and spleen.

It could be shown that the IgM secreting cell number increases, whether T cells are available or not and this is independent of the availability of T cells (Figure 3.26). For IgG and IgA significant differences could be observed. The number of IgG or IgA secreting cells isolated from splenocytes is higher if T cells are available (Figure 3.26).

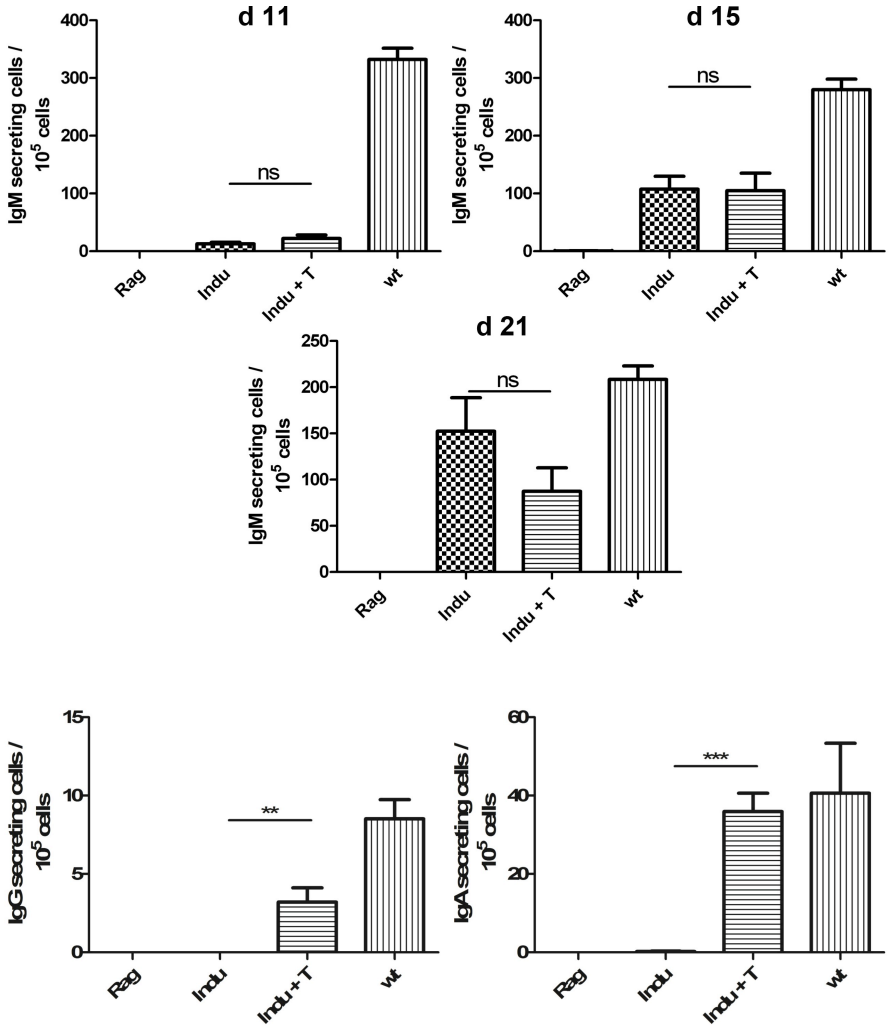

Figure 3.26 Higher number of IgG and IgA secreting cells if T cells are available.

Number of immunoglobulin secreting cells per 10^5 cells for cells isolated from spleens out of the indicated mouse groups are shown. Immunoglobulins are indicated. Time after induction for IgM are day 11, 15 and 21 (as indicated), for IgG day 11 and for IgA day 21 are shown. Stars indicate significances between induced mouse groups with (Indu+T) and without (Indu) T cell administration.

3.3 Functionality of newly developed B-1a cells

As it is still in debate whether B-1a cells can arise from adult bone marrow the B-Indu-Rag1 mouse was created. Here and before (Düber et al., 2009) it could be shown that there are detectable numbers of B-1a cells arising from mice that only have B cell development in adulthood. It was shown, that those B-1a cells are functional, as they spontaneously secrete IgM, show PtC specificity and can survive in $Rag1^{-/-}$ mice after adoptive transfer (Düber et al., 2009). In the present

work it should be analysed, whether these newly developing B-1a cells can compete with those in an established B cell environment. Thereby the aim was to analyse whether it is likely, that B-1a cells are developing from their progenitors also in adulthood if a complete immunoenvironment is present.

3.3.1 B-1a cells developing from adult bone marrow can contribute to the adult B-1a cell pool

To analyse the potency of the adult BM to give rise to B-1a cells when a functional B cell environment is already present, BM chimeric mice were established. For this Rag1$^{-/-}$ or CB20 mice, that express a different IgM allele on their B cells (IgM^{b}) and can therefore be distinguished from B cells arising from B-Indu-Rag1 mice (IgM^{a}), were sublethally irradiated. Subsequently these mice were reconstituted with BM cells isolated from B-Indu-Rag1 mice. Mice were kept for six weeks to establish a functional BM environment and then mice were induced once. 21 days after induction these mice were analysed for their B cell subsets that were derived from the BM of the donor mice by flow cytometry. The main B cell populations in the peritoneal cavity are arising from IgM^{b} progenitors, i.e. cells of the recipient strain (Figure 3.27).

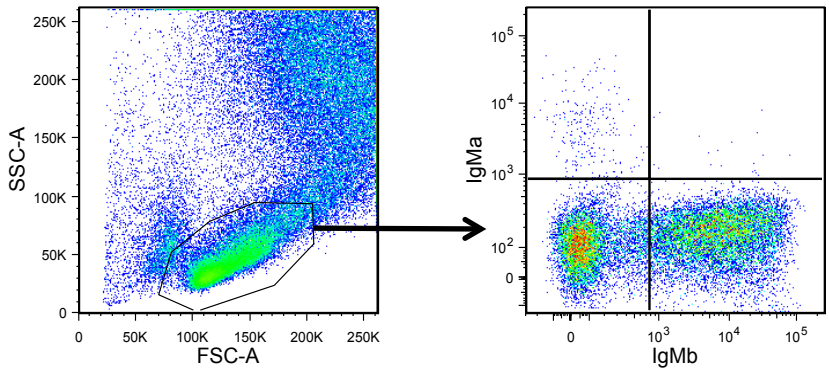

Figure 3.27 Newly developed B cells can be found if need to compete with already developed B cells are found in environment.

Shown is the flow cytometric analysis of peritoneal cavity of a CB20 mouse sublethally irradiated and complemented with BM from B-Indu-Rag1 mice after induction of the B cell development. Doublets were excluded by applying FSC area and heights against each other. Dead cells were excluded by gating for DAPI-negative cells. Cells from recipient and donor mice could be distinguished upon their expression of different IgM allotypes (IgM^{a} donor B cells; IgM^{b} recipient B cells).

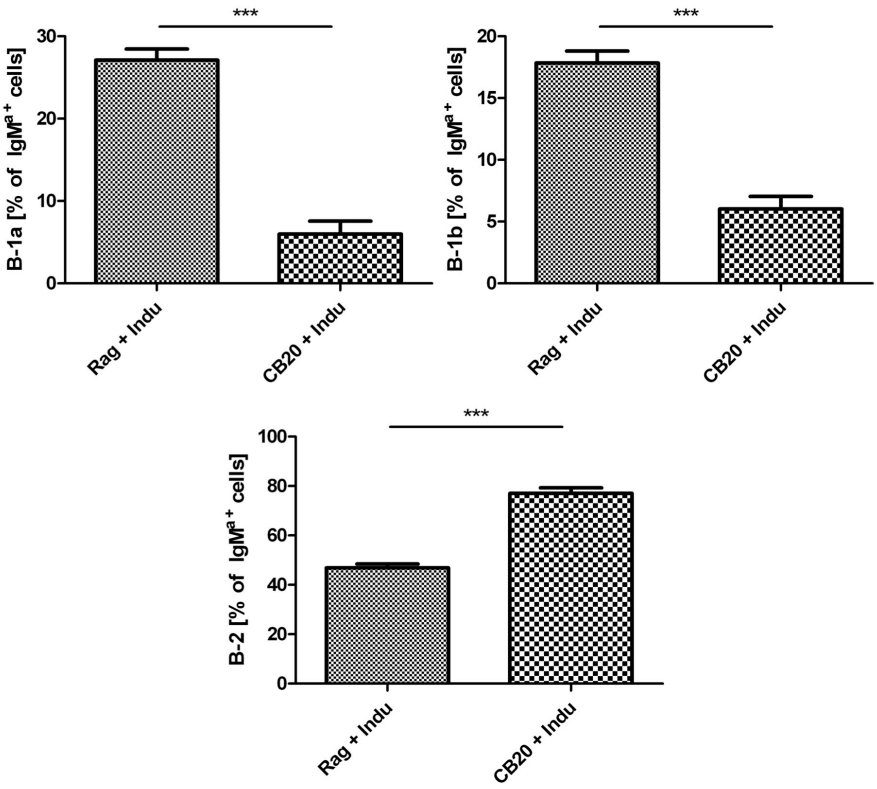

Figure 3.28 B cell development after induction is dependend on the presence of complete immunoenvironment.

CB20 or Rag1$^{-/-}$ mice were sublethally irradiated and complemented with BM from B-Indu-Rag1 mice. After induction of B cell development the appearance of newly developed IgMa^{+} B cells was analysed. Shown are the percentages of B cell subsets from all newly developed IgM^{a+} (donor) B cells in peritoneal cavity.

But, with this experimental setup, it could also be shown that in the peritoneal cavity the donor BM cells (IgM^{a}) give rise to newly developed B cell populations (Figure 3.27). Nearly all newly developed B cells in the peritoneal cavity can be designated as B-2 cells (77 %) indicating that the percentage of B-2 cells from all newly developed IgM^{a+} B cells is higher when there is a complete immunoenvironment existing already. Nevertheless, B-1a cells could be found in the peritoneal cavity of those mice. Nevertheless, the percentage of B-1a cells is lower when B cell development is induced in CB20 (27 % to 6 %; Figure 3.28). In the spleen, also nearly all B cells arose from donor progenitors (IgM^{b+}) (data not shown). Thus, precursors in the adult BM are able to contribute to the adult pool of B1a cells.

3.4 Pentameric or hexameric IgM

Natural IgM are mainly produced by B-1a cells (Baumgarth et al., 2005; Kawahara et al., 2003; Thurnheer et al., 2003). These immunoglobulins are also found in uninfected individuals and mice that are pathogen or even antigen free. It is generally accepted, that B cells are able to secrete pentameric and hexameric IgM. It could be shown, that both isotypes can be found in serum of mice and human (chapter 3.4.1 (Kaveri et al., 2012; Sitia et al., 1990)). Until now, it is not clear which B cell subset produces the hexameric IgM and what is the benefit secreting this isoform. Pentameric IgM consists of five monomeric IgM molecules and contains a joining (J-) chain, while hexameric IgM does not contain a J-chain (Brewer et al., 1994; Davis et al., 1988). In this part of the present work the aim is to figure out, which B cell subset is secreting hexameric IgM.

3.4.1 Normal serum contains pentameric and hexameric IgM

In the present work the appearance of hexameric and pentameric IgM in serum of different mouse strains could be confirmed by isolating IgM by an IgM-specific column from these different sera. After eluting the serum-IgM from the column, it was concentrated and a gradient native PAGE was performed to separate pentameric and hexameric IgM due to their size. The used marker contained purified IgM in both isoforms and was always added in one lane as control. The gel was stained with Coomassie to make the proteins visible. One representative gel is shown in Figure 3.29. It can be seen, that in each serum of the different mouse strains, both, pentameric and hexameric IgM could be detected. This confirms the data of previous findings (Shukala, 2009).

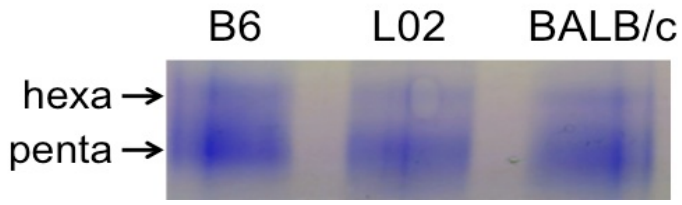

Figure 3.29 Serum of different mouse strains contains hexameric and pentameric IgM.

IgM was isolated from sera of indicated mouse strains and a native PAGE was performed for separating pentameric and hexameric IgM according to their size.

3.4.2 More J-chain expression compared to secretory IgM in splenic B-1a cells but not in peritoneal B-1a cells

It is commonly known that B-1 cells isolated from peritoneal cavity can secrete IgM without stimulation (Holodick et al., 2010; Kawahara et al., 2003; Thurnheer et al., 2003). Nevertheless, the spot size is very small and it was discussed whether these spots may be due to cell lysis or shedding. Recently it was shown by Holodick and colleagues that such spots arise from real secretion from B-1 cells (Holodick et al., 2010). Thus the question arose, which differences may exist between B-1 cells and the other B cell subsets with regard to IgM secretion. For answering the question whether the smaller spot size might be due to different secreted forms of IgM, the J-chain expression of the different B cell subsets and in the same analysis also the IgM expression were analysed. This was done by isolating the different B cell subsets from peritoneal cavity and spleen according to the gating shown in Figure 3.30.

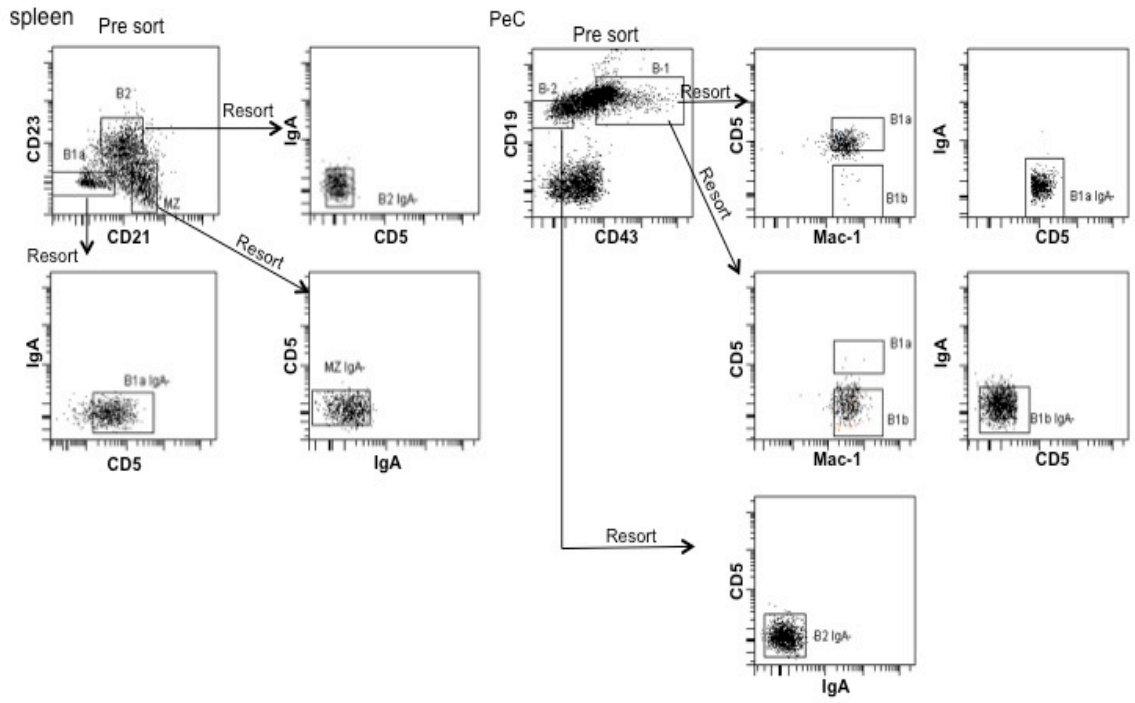

Figure 3.30 Sort for splenic and peritoneal B cell subsets.

The sorting for splenic (left) and peritoneal cavity (PeC, right) B cell populations that are negative for IgA are shown.

The relative expression of different genes from these subsets was analysed by real-time PCR. It could be shown before, and in the present work it could be confirmed, that J-chain is mainly expressed by splenic B-1a cells (Shukala, 2009) Figure 3.31). But, at the same time also the level of expression of secretory IgM is much higher in these cells compared to other B cell subsets (Figure 3.31). In the present work, the ratio of expressed secretory IgM compared to expressed J-chain was calculated as this could give a hint of the usage of J-chain for the assembling of IgM. The level of J-chain expression compared to IgM is much higher in peritoneal cavity compared to that of IgM in splenic B cells (Table 3.1). This led to the suggestion that these cells might give rise to hexameric IgM.

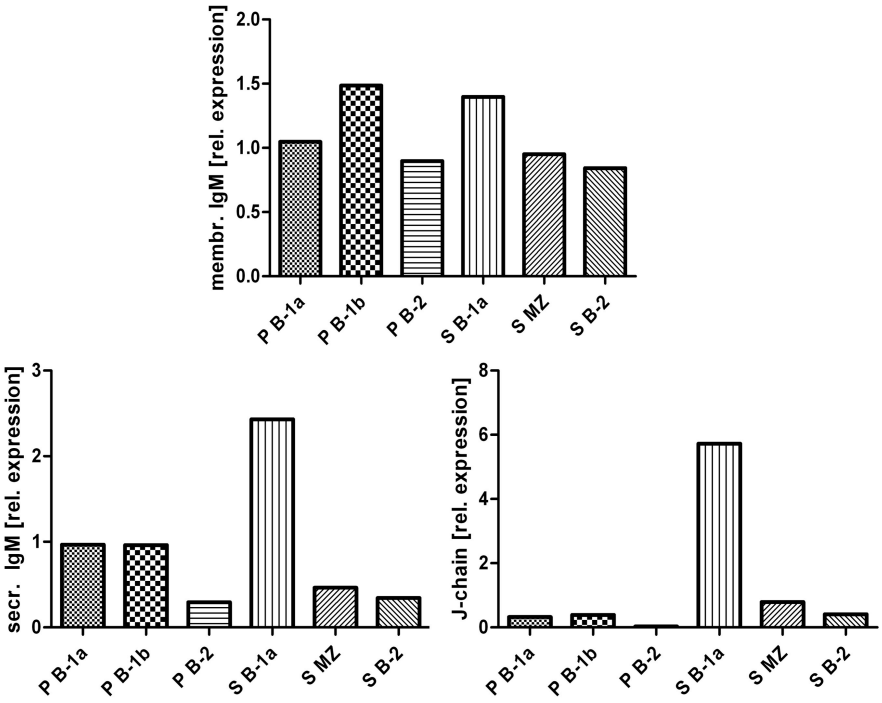

Figure 3.31 IgM and J-chain expression differ between different B cell subsets.

qPCR results for relative expression compared to RPS9 of membrane bound (membr.) -, secretory (secr.) IgM and J-chain are shown. B cell populations and the site of isolation (P=peritoneal cavity; S=spleen) are indicated.

Table 3.1 Ratio of IgM/J-chain is lower in splenic B cells compared to peritoneal B cells

Organ	B cell subset	Ratio IgM/J-chain expression
Peritoneal cavity	B-1a	4.8
	B-1b	2.3
	B-2	4.2
spleen	B-1a	0.6
	MZ	0.8
	B-2	0.6

3.4.3 Transcription factors differ between different B cell subsets

For further analysis of differences between the different B cell subsets that could give a hint for different secretion pattern, the expression of transcription factors and plasma cell marker were analysed. It was suggested that there is a cascade of transcription factors that is found in Ig secreting cells. This cascade includes four different transcription factors: B cell leukemia/lymphoma-6 (BCL-6), B lymphocyte inducer of maturation program 1 (BLIMP-1), paired box gene 5 (PAX-5) and X-box binding protein 1 (XBP-1) ((Tumang et al., 2005) and reviewed in (Calame et al., 2003; Lin et al., 2003)). Tumang and colleagues analysed the expression of these transcription factors in peritoneal B-1a cells, unstimulated and stimulated splenic B-2 cells. The analysis aimed to show phenotypic similarities between B-1a cells and plasma cells (Tumang et al., 2005).

To analyse whether there are differences in the expression of the different transcription factors between all B cell subsets, and to investigate whether one of the unstimulated B cell subsets shows a more plasma cell like expression pattern, the subsets were sorted according to the gating in Figure 3.30 and analysed for their relative expression of three of the transcription factors (Figure 3.32). It could be shown that the expression differs only for PAX-5, that is expressed at higher levels in B-2 and MZ cells. In addition the expression of CD138, a plasma cell marker was analysed. In Figure 3.32 it is shown that it

was highest expressed in splenic B-2 cell subset and lowest for peritoneal B-1a, B-1b and MZ B cells.

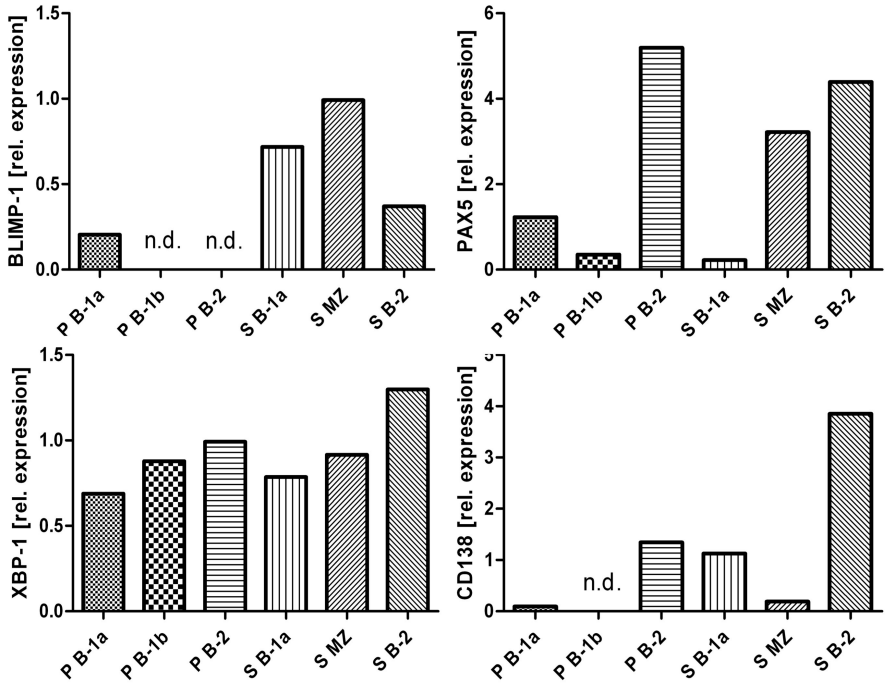

Figure 3.32 Only different expression of PAX5 and CD138 between different B cell subsets.

Shown are the relative expressions compared to RPS9 for the indicated transcription factors (BLIMP-1, PAX-5, XBP-1) or plasma cell marker (CD138). B cell populations and the site of isolation (P=peritoneal cavity; S=spleen) are indicated.

3.4.4 Splenic B cell subsets are not likely to secrete hexameric IgM

As the analysis of transcription factors did not reveal clear significant differences between the B cell subsets, another experiment was performed to establish which B cell subset is secreting hexameric IgM. To this end, different B cell subsets from peritoneal cavity and spleen were sorted, cultured and the supernatants were analysed for secreted IgM. The concentrations of the secreted IgM were expected to be very low in the supernatants of such cultures. LPS stimulation to gain higher concentrations could not be carried out as this induces J-chain expression (Shukala, 2009). Therefroe, radioactive labelled amino acids were added to the medium and IgM was isolated by

immunoprecipitation. The isolated IgM was separated according to their size by a gradient native PAGE. As control, marker with pentameric and hexameric isoform of IgM was added into one lane. Unfortunately most cells died after one day of culture already. Therefore, it was impossible to keep cells for longer than 18 hours. Nevertheless, the amount of secreted IgM still remained low. Thus, it was not possible to unambiguously detect IgM in the supernatant of peritoneal B-1 cell populations. A very faint band was detected for hexameric IgM for peritoneal B-1a cells. Nevertheless it was possible to show that neither splenic B-1a cells nor splenic B-2 cells are secreting hexameric IgM (Figure 3.33).

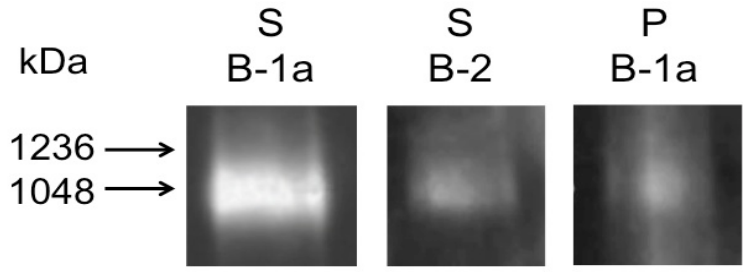

Figure 3.33 Splenic B-1a and B-2 cells secrete pentameric IgM

Different cell populations were sorted and incubated for 18 hours with radioactive labelled amino acids. IgM from supernatants was isolated by immunoprecipitation and a native PAGE was performed for distinguishing hexameric and pentameric IgM by size. Arrows indicate size of molecules at this position given by a marker containing pentameric (1048 kDa) and hexameric (1236 kDa) IgM. S=spleen P=peritoneal cavity.

4 Discussion

The newly established Indu-Rag1 mouse is a highly versatile system to study lymphocyte development. Depending on the Cre recombinase that is crossed into this mouse strain different questions can be answered. For instance, the Indu-Rag1 mouse was crossed to a Rosa26-creERT2 mouse, in which the Cre recombinase is fused to a mutated estradiol receptor and is under the control of the Rosa26 promoter that is ubiquitously active. The induction of the Cre in these mice leads to the continuous development of B and T cells because Rag is activated in hematopoietic stem cells. In recent publications this mouse was used to show that γδT17 cells were not generated after genetic restoration of defective Rag1 function in adult mice (Haas et al., 2012) and that $FoxP3^+$ T cells are commited to a stable lineage that is present already during early stages of murine thymic Treg development (Toker et al., 2013). When the Indu-Rag1 mouse is crossed with MerCreMer mice, in which the Cre recombinase is fused to a mutated estradiol receptor and is under the control of the mb-1 promoter Rag1 will be only expressed in proB cells. This leads to a single wave of maturation because such pro-B cells are not replenished like the stem cells. With this mouse (B-Indu-Rag1) an old puzzle could be unambiguously resolved namely whether adult bone marrow progenitors are able to produce functional B-1a cells (Düber et al., 2009). Additional phenomena that are claimed to occur during B cell development could be proved in the present work.

All results on B cell development in B-Indu-Rag1 mice were gained by several rounds of induction (Düber et al., 2009). Now in the present work, B cell development was induced as a single wave. This single wave made it possible to analyse the minimal time newly developing B cells need for their maturation and for the population of different organs. It turned out that proB cells differentiate into preB cell subsets within five days. The maximum of these two subsets can be detected in the BM seven or 11 days after induction, respectively. The clear order of differentiation could be observed. First the large preB cells develop and later on the small preB cells, as the latter population peaks later as the first one. Small preB cells are known to differentiate from the

proliferating large preB cell population. Interestingly, although the order of differentiation is from large preB II to small preB II such cellular compartments appear at the same time. How can this be reconciled? In the large preB II cell heavy chain rearrangement takes place while in the small preB II cells the light chain is assembled. Large preB II cells that quickly and functionally rearrange their heavy chain should also quickly reach the small preB II state. This would predict that such small preB II cells use mainly the proximal V_H gene segments. This hypothesis can be tested by isolating such cells and establishing the V_H usage.

Table 4.1 Kinetics of B cell development

Shown are the first significant appearances and, when existing the peak of appearance of the indicated B cell subsets isolated from indicated organs. BM=bone marrow; Bl=blood; S=spleen; P=peritoneal cavity.

B cell subset	Detected first [days]	Peak of appearance [days]
large preB II	5	7
small preB II	5	7-11
BM T1	6	11
Bl T1	7	11
S T1	7	11
BM T2	7	11
Bl T2	7	11
S T2	11	11
S B-1a	15	
S MZ	15	
S B-2	11	
BM IgM^+IgD^+	15	
P B-1a	15	
P B-1b	15	
P B-2	11	

Contrary to the clear order observed for preB cell development, such a clear order cannot be detected for transitional B cells. Both B cell subsets (T1 and T2) peak at day 11 post induction. Apparently, the synchrony that is still observed for preB cell development is lost now. However, in contrast to the T1 subset, the T2 subset remains detectable up to day 15 in blood and spleen. The fact that T1 cells disappear first is consistent with the notion of stepwise maturation of transitional B cells and a precursor product relationship between T1 and T2 cells (Allman et al., 2001; Allman and Pillai, 2008). None of the progenitor subsets can be detected beyond 21 days after induction. Clearly only one wave of development takes place and by that time all progenitors have differentiated into mature B cells or have undergone apoptosis as this might also happen due to selection mechanisms.

The first immature IgM^+ B cells (T1 cells) can be detected 6-7 days post induction. This is consistent with data obtained for B cell development in fetal liver. Here, the first c-kit^+ progenitors can be detected at day 11 after fertilization (de Andres et al., 2002). Six days later the first IgM^+ B cells can be detected in the fetus (Raff et al., 1976). This is very similar to what was observed for the BM in the present work, where c-kit^+ cells can be found still at day 0 of induction and the first IgM^+ immature B cells appear 6 days later.

Whereas, after a particular time progenitor and transitional B cells decrease in percentage, the mature B cell populations increase starting with day 11. This indicates that B-2 cells need a minimum of 11 days for full maturation. Again one would predict that such cells are using the proximal V_H gene segements as they have been maturing extremely quickly.

Interestingly, B-1 and MZ B cells appear later than B-2 cells. This could indicate that the differentiation of such B cells requires more time. However, there is no indication for such scenario. More likely is that the precursor frequency for such cells is lower than for B-2 cells. Therefore, it requires more time for these cells to accumulate in significant numbers. Eight weeks after induction, B-1a and B-1b cells can still be found in elevated percentages in peritoneal cavity. In contrast, B-2 cells are lost to some degree. This is coinsistent with the notion

that B-1 cells are self-renewing while the majority of B-2 cells needs to be replenished from precursor cells.

The higher percentage of B-1b cells in induced mice compared to wt mice is consistent with data shown before (Düber et al., 2009). None of the other B cell populations in the peritoneal cavity increases that much. The reason for the differential increase of the B-1b population is still unclear. A possible explanation could be that the recently described precursor for B1 cells in BM leads mainly to B-1b cells (Montecino-Rodriguez et al., 2006).

In the spleen, 15 or 21 days after induction MZ and B-1a cells are found in similar percentages compared to the populations in wt mice. Since B-1 cells are known to be self-renewing (Forster and Rajewsky, 1987; Hayakawa et al., 1986) one would expect that these cells are proliferating to gain their normal level in the different organs. However, no significant increase in proliferating cells was detectable in the induced mice compared to wt mice. One might argue that the BrdU labelling did not work. However, other cell subsets were positive for BrdU (data not shown). Thus, the increase of the population cannot be explained by self-renewal. The expansion of such cells must therefore take place at a level that is not accessible for the analysis carried out here. Most likely the early precursor already proliferate in such a way that the increased numbers can be explained.

The first detection of serum immunoglobulin is on day 15 after induction. By that time also B-1 and MZ B cells can be detected while B-2 cells are found already before. This is consistent with the idea that B-1a cells are the main contributors of secreted IgM. By ELISPOT assays the first IgM secreting cells could be detected by day 11. Apparently this does not lead to a significant increase in secretory IgM. It needs to be shown whether these IgM producing cells are of B-1a or B-2 origin. Both B cells should lead to short lived plasma cells under these conditions (Shapiro-Shelef and Calame, 2005) since no IgM secreting cells could be found in BM at that time points.

Interestingly, other Ig subclasses can also be found in serum by day 21. The production of such Igs is believed to require T cell help. However, the

concentration of such serum antibodies is extremely low. Thus, it can be explained by the leakiness of the immune system that allows also the switch to subclasses other than IgM at a low efficiency.

Despite of the presence of IgG and IgA in serum, no Ig could be detected in the intestinal wash out. This was surprising since the GALT (gut associated lymphoid tissue) is known for mechansims that support a T-independent CSR (Barone et al., 2009; Macpherson et al., 2000). Obviously, IgA secreting cells had not colonized yet the lamina propria of the gut.

To analyse whether T cells have an input on the development of B cells in adulthood, splenocytes isolated from Igα$^{-/-}$ mice containing T cells were injected intravenously one day before induction of B cell development. This should give rise to an environment with T cells that would then be encountered by the newly developed B cells. In a recent publication it was shown that T cells are required in humanized mice for the development of B cells, as they would otherwise rest in the transitional stage (Lang et al., 2013). In the present work it could be shown, this is not the case for mouse B cells. No block at the transitional stage can be observed.

Interestingly, for splenic B-1a cells a T cell effect could be observed. The numbers are significantly increased in mice that received adoptively transferred T cells compared to those that were induced without T cells. In contrast, peritoneal B-1a cells appear to be completely independent on T cells. No increase in the cell number could be observed. The reason for this T cell effect in the spleen is unclear. It is known that B-1a cells react to their specific antigen in a T-independent manner (Yang et al., 2012). In addition, the differences between B-1a cell populations from spleen and peritoneal cavity are still ill defined. Possibly, migration of B1a cells to the spleen is supported by T cells. This has been observed in our group before (Roy et al., to be published).

T cells also have an impact on MZ and B-2 cells in the spleen. The findings might indicate that T cells are important especially for the early differentiation or maintenance of B-2 cells. They are found in higher numbers after three weeks of induction. At later time points this difference is no longer seen. Therefore T

cells might support a faster development of B-2 cells in the spleen, as this increase in number is also found in peritoneal cavity.

The impact of T cells on MZ B cells appears to act mainly on maintenance because their numbers are increased at 8 weeks quite in contrast to B2 cells.

To investigate how much the function of B cells after induction is influenced by the presence of T cells, the production of immunoglobulins was analysed. It is known that the cytokine milieu that is at least in part dependent on T cells, acts on the type of CSR during activation of B cells (Manis et al., 2002; Stavnezer et al., 2008). Thus, the appearance of some of the immunoglobulin subsets is highly dependent on the presence of T cells (Lentz and Manser, 2000). Therefore, the immunoglobulin concentrations in the serum of induced mice that received adoptively transferred T cells were compared to sera of induced mice without T cell. After 21 days (at this time point the first significant amounts of immunoglobulins could be detected in only induced mice) also the concentration in the serum of those mice that have T cells available was higher compared to those without T cells. This was consistent with the finding that there are more immunoglobulin secreting B cells in mice that received T cells. Even for intestinal wash out it could be shown that the IgA secretion is highly dependent on such T cells (Barone et al., 2009). Obviously still a high number of B cells do require T cells for the switching to IgA-secreting cells and possibly also for migration.

The higher serum concentration of immunoglobulins in mice that received T cells is lost at later time points, except for IgA that remains higher. Obviously T cells are required for activation and switching of the B cells that newly develop but only short lived plasma cells develop under these conditions except for IgA. This might be due to the continuous stimulation of the immune system by commensal bacteria that are located in the gut of SPF mice. These stimuli are supposed to activate B cells in a T cell-independent manner (Macpherson et al., 2000), but obviously this is not sufficient for an increase in IgA concentration, neither in intestinal washout, nor in serum.

T cells definitively help B cells in maturating to immunoglobulin secreting cells. This could be confirmed by ELISPOT. The number of immunoglobulin secreting cells is increasing for IgG and IgA, when T cells are available. For IgM no increase could be shown. This might demonstrate the T cell independence of IgM secreting cells

It was for long time in debate whether adult BM can give rise to B-1a cells or whether this is an exclusive property of fetal progenitors. Here and before (Düber et al., 2009) it could be shown that also from adult BM B-1a cells can develop. By analysing their ability to bind PtC and spontaneously secretion of IgM it could be shown that these B-1a cells are functional (Düber et al., 2009). As these B-1a cells are developing in an immunodeficient environment it could be still argued that the adult BM might not give rise to these B-1a cells when a complete and functional immunoenvironment is present. In addition, it was not formally shown that B-1a cells derived from adult BM are contributing to the adult B-1a cell pool. Therefore a BM chimera was established in which B cell development could be induced in a “normal” environment. B-1a cells could derive from these newly induced B cell progenitors. In addition, it could be shown that the progenitors more likely develop into B-2 subset than into B-1 subsets when other B cells are available. Why this might happen and what regulates progenitors to become B-2 cells rather than B-1a cells when a complete immunoenvironment is available has to be answered. Obviously the B-1 cell niche is saturated already in these immuncompetent mice and the number of B-1 cells does not rise above a certain level. Possibly, as soon as the B-1 cell niche in the periphery is filled a feedback to the BM takes place and no more cells will differentiate into B-1 cells. The still high differentiation into B-2 cells might be due to the fact that B-2 cells are short lived compared to B-1 cells and therefore need more replacement and renewal. The fact, that in some transfer experiments, no newly developed B-1a cells could be found (Kantor and Herzenberg, 1993) might be due to the fact that the percentage of newly developed B-1a cells is very low and it might be taken that no new development of these cells takes place. It was shown before that B-1 cells are newly developing from adult bone marrow (Düber et al., 2009; Holodick et al., 2009),

but in these cases the newly developed cells did not have to compete with a functional immunoenvironment. In the present work it could be clearly shown that also in immunocompetent mice B-1a cells do develop from adult BM-progenitors and contribute to the adult B-1a cell pool to some extend.

It is known that in serum IgM can be detected in pentameric and hexameric isoform (Kaveri et al., 2012; Sitia et al., 1990), whereas pentameric IgM is found in much higher concentrations (Brewer et al., 1994; Shukala, 2009). To investigate whether a specific B cell subset is secreting hexameric IgM, the relative expression of secretory IgM and J-chain was compared. It could be shown that the ratio of IgM to J-chain is much higher in peritoneal B cell subsets compared to the splenic B cell subsets. Thus, the peritoneal B cells might secrete hexameric IgM as this isoform does not contain J-chain. And as the highest expression of IgM compared to J-chain was found in peritoneal B-1a cells these cells were thought to be most likely cells that are secreting this isoform. By analysing the supernatants from sort-purified B cell subsets it could be excluded that splenic B-1a and splenic B-2 cells are the cell subsets that are secreting hexameric IgM. Due to very low IgM secretion of peritoneal B cells, the analysis of these subsets did not yield conclusive results. The native PAGE analysis revealed a minor band for hexameric IgM for supernatants from B-1a cells. Nevertheless, ambiguity remains for technical reasons.

To investigate further differences between the different cell subsets, it was analysed whether the expression of transcription factors that are acting in a cascade in Ig secreting cells (Tumang et al., 2005) might differ between the different cell types. This should give a hint on differences between the different cell subsets. The only differences could be observed in PAX-5 expression. This transcription factor is normally found in elevated levels when B-2 cells are non-secreting (Tumang et al., 2005). Thus, B-1 cells display a more secretory phenotype. Unfortunately the expression patterns for the other transcription factors do not fit to that finding. XBP-1 and BLIMP-1 did not differ between these subsets. In addition CD138, a marker for plasma cells, was elevated in splenic B-2 cells. Therefore, the analysis of the relative expression of neither of the transcription factors, nor of CD138 answers whether B-1a cells are the hexamer

secreting cell subset or not. Therefore in the present work it could be shown that peritoneal B-1a cells secrete pentameric as well as hexameric IgM. Unfortunately the evidence is not completely compelling.

Previous studies showed that in young adult bone marrow cells the proximal V_H genes are used more often (Malynn et al., 1990). This could be confirmed by using the B-Indu-Rag1 mouse. In the first days of B cell development the proximal genes, close to DJ are used and at later days the distal (J558) genes were used in higher frequency. As the J558 gene family is a large family it could be argued that simply more genes are used over time. On the other hand the clear shift from proximal to distal gene usage can be seen as there are by day seven post induction around 40 % of gene segments used from the J558 family, whereas it is only 10 % by day 1-3. Apparenty, an individual B cell precursor has the chance to rearrange a proximal V_H gene segment first. As the process is slow, only some cells will be able to successfully assemble a V_H gene. These cells most likely finish maturation with minimal times. Cells that require more time for rearrangement have then the complete repertoire available, since the locus is now completely open due to cytokine activity or other not yet defined signals.

As in normal wt mice, the B cell development is not synchronized this could not be clearly found in earlier experiments. It was also shown before that isolated B cell precursors that were isolated from $Rag2^{-/-}$ mice the histone assembly and therefore the active recombination with these genes was highly dependent on IL-7 for the distal genes (Chowdhury and Sen, 2001). In the present mouse model, it would be possible to investigate this *in vivo*. IL-7 given systemically at the time of induction should result in activation of distal genes to be used for assembling V_H genes in the first days of B cell development.

The present work adds new aspects that are addressed by using the B-Indu-Rag1 mouse. Thus, this mouse model offers a highly versatile tool to investigate early steps of B cell development *in vivo* as the first differentiation steps are synchronized. Similarly, aspects of T cell development can be investigated using appropriate combination of inducible Cre.

5 Summary

For the present work the B-Indu-Rag1 mouse has been used to investigate the kinetics of B cell development in adult mice. In this mouse, Rag1 which is required for T and B cell development is inverted and flanked by inverted loxP sites. When crossed with a mouse that bears a Cre that is fused with a mutated estradiol receptor under the control of the B cell-specific mb1 promoter, expression can be induced by administration of Tamoxifen. This leads to one wave of B cell development.

Uninduced mice contain a pool of c-kit$^+$ proB cells. Such cells can differentiate upon Tamoxifen induction. Thus, the time that B cells and their precursors need for maturation can be determined. ProB cells need five days to differentiate into preB cells and it takes another two days to detect the first transitional B cells in different organs. This is in agreement with the development observed in fetal liver. The progenitor and the immature B cells peak at a certain time point which is consistent with the notion that the induction under these circumstance leads to a single wave of B cell development.

Using the synchrony at the early time points after induction, it was possible to demonstrate that during heavy chain recombination in progenitor B cells the V_H genes proximal to the DJ segments are used first and the distal V_H segments are rearranged later during B cell ontogeny.

Analysis of the mature B cell populations revealed an earlier appearance of B-2 cells compared to B-1 and MZ B cells. This could be simply due to the precursor frequency for such B cells which might be higher for B-2 cells. Importantly, no proliferation could be found neither for transitional nor for mature B cells. Apparently, expansion takes place only at the precursor level.

Using BM chimera it could be shown that B-1a cells developing from adult progenitors contribute to some extend to the B-1a cell pool in adult mice.

In addition the source of hexameric IgM was analysed. Peritoneal B-1a cells appear to secrete pentameric and hexameric IgM, while splenic B cells clearly do only secrete pentameric IgM.

The present work adds compelling data for the development and function of B cells in adult mice. Furthermore it shows that the B-Indu-Rag1 mouse model is a highly versatile tool to investigate questions regarding development of B cells in particular and lymphocytes in general.

6 Appendix

6.1 Abbreviations

Ab	antibody
AID	activation-induced cytidine deaminase
APC	Allo-Phyco-Cyanin
BCR	B cell receptor
Bio	Biotin
BM	bone marrow
bp	base pairs
C	constant region gene segment
CD	Cluster of differentiation
cDNA	complementary DNA
CDR	complementary-determining region
CLP	common lymphoid progenitor
CSR	class switch recombination
Cys	cysteine
D	diversity gene segment
DAPI	4′,6-Diamidin-2-phenylindol
DMF	dimethyl-floramid
DNA	deoxyribonucleic acid
DSB	double stranded DNA breaks

EDTA	ethylene-diamine-tetra-acetate
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immuno spot assay
et al.	et alii
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FO	follicular
FSC	forward scatter
H	heavy
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
i.e.	id est
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
J	joining gene segment
J	joning
L	light
Mb	mega bases
Met	methionine
MHC	major histocompatibility complex

mIg	membrane Ig
mRNA	messenger RNA
MZ	marginal zone
PBS	phosphate buffered sline
PCR	polymerase chain reaction
PE	phycoerythrin
PHSC	pluripotent hematopoietic stem cell
pre	precursor
pro	progenitor
Rag	recombination activating gene
RNA	ribonucleic acid
RT	reverse transcription
RT	room temperature
sIg	surface immunoglobulin
SL	surrogate light (chain)
SPF	specific pathogen free
T	transitional
TdT	terminal deoxynucleotidyl transferase
wt	wild type

6.2 References

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